The Role of Auxin in Host Responses During Cauliflower Mosaic Virus Infection

By

Lorna Ann Smith

A thesis presented for the degree of Doctor of Philosophy

In

The Institute of Biomedical and Life Science
Division of Biochemistry and Molecular Biology
At the University of Glasgow

October 2006

Declaration

I hereby declare that the work submitted in this thesis is the result of my own investigations except where references are mentioned and assistance is aknowledged. Therefore no part of this thesis has been previously presented for any degree.

Lorna A. Smith

October 2006

Dedication

To all who have supported me.

Acknowledgements

I would like to thank all who have supported and guided me throughout my studies. In particular I would like to thank my supervisor Dr Joel Milner who has offered invaluable advice and shared his knowledge and expertise with me. I would also like to acknowledge the help and support of all of the members of the Wolfson lab, especially Dr Love for his encouragement and guidance. This work would also not have been possible if it was not for the technical support offered by our technician Janet Laird.

Publications

Smith L. Love A. J, Laird J., Armenguad P., Milner J. J. (2006) Cauliflower mosaic virus-infection and auxin signaling in Arabidopsis, Advances in Plant virology, University of Warwick, Poster 11.

Table of Contents

Chapter Two

Materials and Methods

List of Tables

List of Figures

Abbreviations

AAAP : Auxin amino acid permease

ABA : Abscisic acid

ACMV : African cassava mosaic virus

ATF : Aphid transmission factor

AOX : Alternative oxidase

ATP : Adenosine triphosphate

BCTV : Beet curly top geminivirus

BYDV : Barley yellow dwarf virus

CaMV : Cauliflower mosaic virus

CMV : Cucumber mosaic virus

CP : Coat protein

dCTP : Deoxycytosine 5'-triphosphate

dNTP : deoxyribonucleotide triphosphate

dpi : Days post inoculation

EACMV : East African cassava mosaic virus

GA : Gibberellic acid

GFP : Green Fluorescent Protein

GLM : General Linear Model

GUS : β-glucuronidase

HR : Hypersensitive Response

IAA : Indol-3-acetic acid

JA : Jasmonic acid

LB : Luria-Bertani media

MeJA : Methyl jasmonate

MP : Movement protein

MS : Mass spectrometry

NLS : Nuclear localization signal

NPA : 1-naphthaleneacetic acid

ORF : Open reading frame

PCR : Polymerase Chain Reaction

PD : Plasmodesmata

PAT : Polar auxin transport

PAV : Barley yellow dwarf virus strain PAV

PPV : Plum pox virus

PR : Pathogenesis-related

PVM : Potato virus M

QPCR : Quantitative poymerase chain reaction

RdRp : RNA dependent RNA polymerase

ROS : Reactive oxygen species

RT-QPCR : Reverse trasnscriptase quantitative polymerase chain reaction

SA : Salicylic acid

SAR : Systemic Acquired Resistance

SEL : Size Exclusion Limit

TAV : Transactivator domain

TBSV : Tobacco bushy stunt virus

TCV : Turnip crinkle virus

TGB : Triple gene block

TIBA : Triiodobenzoic acid

TMV : Tobacco mosaic virus

ToMV : Tomato mosaic virus

TRSV : Tobacco ringspot nepovirus

TSV : Tobacco streak virus

TSWV : Tomato spotted wilt virus

UBQ : Ubiquitin

Abstract

The role of auxin in the host response during compatible virus infection was examined in Arabidopsis with Cauliflower mosaic virus (CaMV). A genetic approach was adopted and a number of auxin signalling and transport mutants were used.

The host responses including, symptom type and severity, virus movement and virus titre were not affected by disruptions to auxin signalling conferred by mutations of the *AUX1* influx carrier or by mutations in *TIR1* or *TIR2*. The *tir3-1* mutation which contains a mutation in *TIR3* locus which is involved in the synthesis, localisation and functioning of the NPA binding site within the efflux carrier as well as protein trafficking did show an altered response to infection. *tir3-1* plants showed a consistent delay in the development of symptoms as well as delayed and reduced virus titre and viral spread.

Virus infection up-regulated the expression of a number of auxin responsive genes, *SAG21*, *SAUR_e* and *GH3*. This upregulation was significantly reduced in *aux1-100* and *tir3-1* mutants. The expression of another auxin responsive gene *LAX2* was found to be reduced in response to infection. From these findings it would appear that auxin signalling and transport is involved in some way during the infection process.

Auxin levels were quantified in the *AUX1* mutant allele *aux1-100* and the wild type WS. No differences in levels of free IAA were observed. A synthetic auxin response element was also examined using both GUS and GFP reporters

but again no alterations in auxin could be found. This could be due to the changes in auxin concentration being very subtle and therefore the techniques used failed to identify these changes, or it could be that the virus is interacting with specific components of the auxin signalling and transport pathways and the auxin responses are as a consequence of this rather than direct changes in auxin levels.

One CaMV gene product which may be interacting with the auxin pathway is P6. Previous studies have shown that P6 is responsible for symptom determination and host response and that it interacts with other signalling pathways including the ethylene and SA pathways. It has been found here that P6 confers resistance to the auxin efflux inhibitor TIBA and that it increases the expression of the auxin responsive gene *SAUR_e*. This resistance is independent of the chlorosis and stunting associated with P6. Preliminary genetic analysis of this interaction has idendified an interaction between *tir3-1* and P6. Progeny of crosses between *tir3-1* mutants and the P6 transgenic Arabidopsis line A7 show an abernat segregation ratio which suggests that *tir3-1* is able to suppress the P6 phenotype.

These results suggest that a specific component of the auxin signalling pathway is involved in the host response to compatible infection. It is possible that P6 interacts directly with *TIR3* rather than altering IAA levels. *TIR3* is involved in protein trafficking and therefore could be a requirement for successful infection. Alterations in this gene could affect the trafficking of auxin signalling

components such as PIN1 and lead indirectly to the auxin responses seen during infection.

Chapter One

Introduction (1.1)

Plants are vulnerable to attack by a variety of different pathogens, including bacteria, fungi and viruses, and so must therefore continually defend themselves against infection. The effects of bacterial and fungal pathogens on plant growth and the pathogenic effects of plant viruses on plant growth, development and symptom production are well documented in plant literature but the mechanisms by which plant viruses cause these effects are less well understood at a molecular level.

The speed and pattern at which a virus moves in nature depends on the relationship between the virus and the vector, on the numbers and activity of the vectors and on many other factors (Hull, 2001). Some viruses under specific conditions may infect a plant without producing any obvious signs of disease whilst others may lead to the rapid death of the whole plant. One of the main reasons for studying plant viruses is the impact that the diseases they cause have on crop productivity worldwide. It has been suggested that at least 10% of global food production is lost due to plant disease (Strange and Scott, 2005). Viruses are of economic importance when they cause some significant difference in the growth of the host plant compared to a normal uninfected plant, or when they reduce the quality of the crop. It is difficult to obtain accurate data on the scale of the losses caused each year by viruses, as these losses are commonly less obvious than those caused by other plant pathogens and tend to last for much longer. Barley yellow dwarf virus (BYDV), for example, is distributed worldwide and infects over 150 species of the Poaceae, including the majority of

the staple cereals, such as wheat, rice, barley, oats and maize (Strange and Scott, 2005). A number of studies have demonstrated the debilitating effect these viruses have on cereals, for example, grain yield of maize infected with the PAV serotype of the virus was 15-20% less than in uninfected controls because the infected plants produce fewer kernels per ear (Beuve *et al.*, 1998). Whereas barley infected with the same serotype showed reductions in yield up to 38% (Edwards *et al.*, 2001).

Cassava is a very important food crop for farmers particularly in low-income, food deficit countries. However, it is prone to diseases caused by mosaic viruses. The sensitivity of the crop to such viruses as African cassava mosaic virus (ACMV), East African mosaic virus (EACMV) and a more severe recombinant virus called UgV, and the extent of the losses caused by these isolates, has lead to many farmers abandoning cassava cultivation (Zhou *et al.*, 1997). This has serious consequences for food production and security in the African countries affected.

It is the physical symptoms of disease e.g. stunting, vein clearing and chlorosis that cause the loss of viable crop plants and hence lead to economic losses (Gibbs and Harrison, 2002). Therefore it is necessary to investigate the causes of these symptoms of disease because it is only once we understand the causes that we will be able to start addressing the issue of how we can control them. The ability to control the development of symptoms during viral infection would allow a better understanding of how to reduce the economic losses that are

caused by viral diseases throughout the world. A detailed study of plant-microbe interactions will also allow us to elucidate the signalling mechanisms by which plant cells deal with a stress situation. In order to study the effects of plant viruses on host species model systems have been exploited using viruses that are well characterised and host plants that are equally well understood. One such system is the Caulimovirus, Cauliflower mosaic virus (CaMV) and *Arabidopsis thaliana* (Arabidopsis).

Symptoms (1.2)

When a host plant is infected with a virus either a compatible or incompatible interaction may occur. Recognition of a genetically incompatible pathogen results in the activation of a complex series of defence responses (DeWit, 1997; Dangl and Jones, 2001). The process is co-ordinated both temporally and spatially to ensure that only the necessary numbers of plant cells are recruited from primary metabolism into a defensive role (Hull, 2001; Hammond-Kosack and Jones, 2000). This rapid and highly localised induction of plant defence response results in the creation of unfavourable conditions for pathogen growth and reproduction, at the same time, the responding cells detoxify and impair the spread of harmful enzymes and toxins produced by the pathogen. Full activation of this intense response to the pathogen generally occurs within 24 hours and usually leads either directly or indirectly to localised cell and tissue death known as the Hypersensitive Response (HR; Heath, 2000; Shirasu and Schulze-Lefert, 2003) and eventually localised and systemic resistance (Hull, 2001; Dangl *et al.*,

1996). A compatible reaction however, is one in which the pathogen can spread throughout the plant and cause a systemic infection. During compatible interactions the expression of specific host and viral genes leads to the development of viral disease and the symptoms associated with such disease.

The fact that both viruses and viroids induce disease symptoms demonstrates that nucleic acids themselves, as well as proteins, are sufficient to cause symptoms. However, the severity of symptoms is not necessarily related to pathogen titre, indicating that disease is often the result of specific interactions and not general distress (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002; Lee *et al.*, 1996). For example, a spontaneous mutant of Tobacco streak virus (TSV) that has a single nucleotide change in the intergenic region of RNA3 induces more severe and prolonged symptoms in tobacco than the parental virus, despite a lower titre (Xin and Ding, 2003).

The symptoms associated with disease differ greatly depending on the virus, host plant and environmental conditions (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002). Leisner *et al.*, (1992) demonstrated in CaMV infection of Arabidopsis that if the development of early flowering plants is retarded by growth under sub-optimal conditions inoculated plants become more susceptible to the virus and systemic infections more widespread.

Local symptoms tend to include localised lesions that develop near the site of entry, a loss of chlorophyll, and the development of necrotic lesions where

infected cells have died (reviewed by Hull, 2001). Often these localised lesions are not of any great economic significance. Systemic infection however, is of much more importance economically and can induce a range of symptoms depending on the virus and the host plant. Infected plants often show common features such as, leaf yellowing, leaf distortion and/or many other growth distortions. Stunting is common during viral infection and may affect the whole plant or certain parts of the plant more than others (reviewed by Hull, 2001). This is generally due to a reduction in leaf size rather than a reduction in the number of leaves that develop. Reductions in the total yield of fruit is common as well as a reduction in the quality of the fruit produced which again is of major economic significance e.g. with Plum pox virus (PPV; Németh, 1994; Dunez and Sutic, 1988). One of the most frequent and most obvious effects of virus infection is the development of mosaic patterns in infected leaves. Vein banding can vary in its severity, with some plants showing very faint banding and others much more striking emphasis to the vein pattern. This feature of virus infection can often persist as a major symptom of the disease. Symptoms of disease are often similar to those produced by a wide range of physical, chemical and biological agents, such as, hormone treatment and temperature changes, which indicates that it is changes within the host plant that leads to the physical symptoms of disease. It is thought that virus infection activates genetically pre-programmed responses in the host plant (Covey *et al.*, 2000; Johal *et al.*, 1995) resulting in the development of specific symptoms.

Plants can show a variety of responses to pathogens and in the case of susceptible plants they usually develop the symptoms of disease. An additional host response to viral infection is sometimes characterized by the initial development of the typical symptoms associated with the infection, followed by a recovery of the host plant (Chellappan *et al.*, 2004). This recovery is due to inactivation of the virus by a host response reminiscent of cosuppression of transgenes and posttranscriptional gene silencing (Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Al Kaff *et al.*, 1988). It was demonstrated that when Kohlrabi (*Brassica oleracea gongylodes*) was infected with CaMV it initially developed systemic symptoms but within weeks it had recovered through a loss of the virus. At 14 or 15 days after infection, a few days before the amelioration of the plant symptoms, there was a sudden transition in the composition of viral DNAs (Al Kaff and Covey, 1995). Reverse transcription products were lost, whereas supercoiled DNA was amplified. These changes are consistent with a sudden arrest of viral replication by gene silencing. To escape the gene silencing defences of plants, viruses have developed a counter defence strategy. Many plant viruses encode suppressors of gene silencing (Moissard and Voinnet, 2004; Chapman *et al.*, 2004; Voinnet *et al.*, 1999), as do some animal and insect viruses (Li *et al.*, 2002). For example the CMV 2b protein of Cucumber mosaic virus (CMV; Ji and Ding, 2001), HC-PRO of Potyviruses (Brigneti *et al.*, 1998; Zhao *et al.*, 2005) and P19 of tombusviruses (Silhavy *et al.*, 2002; Qui *et al.*, 2002) are associated with the enhancement of viral pathogenicity and virus accumulation.

Movement (1.3)

To induce a disease, a virus has to be able to spread from the site of initial infection throughout much of the rest of the plant and replicate. Over recent years the understanding of how viruses move within plants has greatly increased, and has led to the identification and characterization of virus encoded proteins that are involved in virus movement from cell to cell, known as movement proteins or MPs, as well as other factors that control movement. It was first suggested by Samuel (1934) that virus movement could be divided into two different components, cell to cell movement and long distance movement. Cell to cell movement usually refers to the movement from the initially infected cell or cells, usually epidermal or mesophyll cells, to the vascular bundle. Once a virus arrives at the vascular tissue long distance movement begins and the virus is transported through the vasculature, usually through the phloem sieve tubes to the rest of the plant. Then further cell to cell movement establishes systemic infection. In order for plant viruses to move from cell to cell within their hosts they have had to overcome the barrier that the cell wall creates. To do this, plant viruses, have evolved to move between cells directly through intercellular connections, the plasmodesmata (Hull, 1989; Lucas and Gilbertson, 1994). Plasmodesmata are cytoplasmic connections between adjacent cells and they play important roles in intercellular transport, cell to cell communication, cell differentiation, plant growth and development (Gunning and Robards, 1976; Boevink and Oparka, 2005). The smallest physical dimension of a known plant

virus is larger than 10 nm (Gibbs, 1976) whilst a recent high-resolution electron microscopic study established that the most obvious transport channels within plasmodesmata of tobacco leaves are physically about 2.5 nm in diameter (Ding *et al.*, 1992). Therefore, plant virus particles are too large to be able to pass through unmodified plasmodesmata, so it is believed that this transport likely occurs by an active mechanism mediated by specific viral movement proteins (Oparka, 2004; Oparka and Roberts, 2001).

The first proposed movement protein was identified by Nishiguchi and colleagues (1978) who showed that the temperature sensitivity in the capacity of Tomato mosaic virus (ToMV) to move through its host was not due to any effect on replication and was subsequently mapped to the 30 KB pp gene. This then led to the identification of the 30-kDa movement protein of tobamoviruses (Deom *et al.*, 1987), and since then, to the identification of MPs in many other plant viruses. It is thought that different virus MPs act in different ways to facilitate the spread and movement of the virus through the host plant (Carrington *et al.*, 1996).

Four virus families including the caulimoviridae produce tubules that extend through the plasmodesmata or cell walls. In the case of the caulimoviridae it has been shown that these tubules contain virus particles. Carrington *et al* (1996) suggested that MPs localize to the plasmodesmata where they induce the removal of the desmotubule and assemble into tubules that extend unidirectionally into the adjacent cell. Virions that have been assembled in the

cytoplasm are then escorted to the tubules through interactions with their MP and subsequently transported to the adjacent cell (Perbal *et al.*, 1993; Storms *et al.*, 1995). Other MPs act as chaperone molecules, which bind to the viral nucleic acid and form an extended and unfolded protein-nucleic acid complex that targets modified plasmodesmata (Carrington *et al.*, 1996). This is thought to be how Tobacco mosaic virus (TMV) MP functions (Citovsky, 1999). TMV MP interacts with the plasmodesmata and modifies the gating properties and therefore directly facilitates the movement of viruses through the plasmodesmata (Ding *et al.*, 1992; Wolf *et al.*, 1989). It is thought that MPs also facilitate the active transport of genomes from cell to cell rather than merely increasing the Size Exclusion Limit (SEL) (Citrovsky *et al.*, 1990; Citovsky and Zambryski, 1991). BL1 MP of bipartite geminiviruses and the product of ORF 2 of potato virus X are also believed to act in this way (Carrington *et al.*, 1996).

A final group of plant viruses exploit a unique triplet of proteins known as the triple gene block (TGB) of movement proteins to facilitate virus spread and establish a systemic infection in the host plant (Waigmann *et al.*, 2004; Hull, 2001). The three proteins work together to transport the virus RNA from the initial infected cell, through surrounding cells to the vascular system for translocation throughout the plant (Morozov and Solovyev, 2003). Although some information is available on the relative roles of the TGB proteins, the molecular details of the processes are not well understood. A combination of molecular and cell biology tools have been used to try to dissect the mechanisms involved (Lough *et al.*, 1998; Torrance *et al.*, 2006; Tamai and Meshi, 2001).

The phloem of higher plants is an essential pathway for the movement of photoasimilates from mature, exporting tissues often referred to as sources, to immature, developing tissues that exert a carbon demand and therefore act as sinks (Gifford and Evans, 1981; Roberts *et al*., 1997). Phloem transport and unloading within intact plants has been confirmed through the use of confocal laser scanning microscopy (Oparka *et al*, 1994; Wright *et al*, 1996). It has been shown that plant viruses also have the ability to move over long distances in the phloem (Maule, 1991; Leisner and Turgeon, 1993; Roberts *et al*., 1997). This process of long-distance transport is less well understood than that of cell to cell transport and has very different requirements from those of cell to cell movement (Ryabov *et al*., 1999). Minor veins are generally sheathed by bundle sheath cells and contain various cell types including vascular parenchyma cells, companion cells and enucleate sieve elements (Nelson and van Bel, 1998). Therefore, transport of a virus to and within vascular tissue implies movement from mesophyll cells to bundle sheath cells, from bundle sheath cells to vascular parenchyma and companion cells, and entry into sieve elements (Ryabov *et al*., 1999). It is unclear how or where these viruses exit the phloem, but it could be in the reverse order to that described. It has been shown, that coat protein (CP) is essential for efficient long-distance movement of most plant viruses (Petty and Jackson, 1990). However, CP is not required for cell to cell movement of TMV but it is essential for long-distance movement (Ryabov *et al*., 1999). In the few cases where the CP gene is partially or entirely dispensable for systemic spread, the time required for systemic spread may be greatly increased in its absence (Scholthof *et al*., 1995). Many viruses also encode

proteins that provide additional functions required for systemic spread (Ryabov *et al.*, 1999). Tobacco bushy stunt virus (TBSV) encodes two structural proteins, p19 and p22, both of which are necessary for systemic infection. Cell to cell movement functions are provided by p22, whereas p19 promotes long-distance transport in a host specific manner (Scholthof *et al.*, 1995). CMV also encodes a protein, 2b, that promotes host specific long-distance movement (Ding *et al.*, 1995; Ji and Ding, 2001) and potyvirus HC-Pro protein provides functions required for both long-distance movement and efficient genome replication (Klein *et al.*, 1994; Cronin *et al.*, 1995). CMV 2b, the HC-Pro of potyviruses and P19 of TBSV are all suppressers of gene silencing, thus implies a link between long distance movement and silencing.

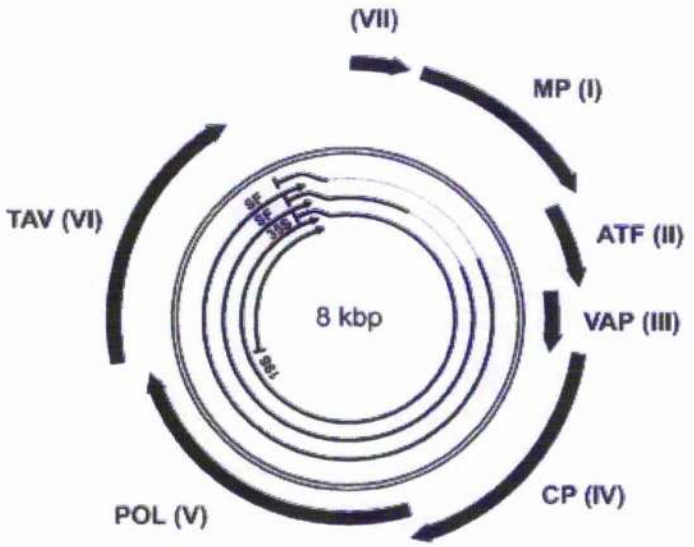
There is also some limited genetic evidence that MPs perform specific long-distance movement functions in addition to their roles in cell to cell movement (Feneziik *et al.*, 1995; Wang *et al.*, 1998). For example, several alanine-scanning mutants of Red clover necrotic mosaic virus (RCNMV) with substitutions affecting the MP exhibit host-specific defects in long-distance transport (Carrington *et al.*, 1996). Additionally, many viruses encode replication proteins that appear to have specific roles in long-distance transport (Weiland and Edwards, 1996; Ding *et al.*, 1995; Treynor *et al.*, 1991; Nelson *et al.*, 1993).

Cauliflower mosaic virus as a model system (1.4)

A useful model system for showing the interaction between a higher plant and a compatible viral pathogen is Cauliflower mosaic virus and the plant Arabidopsis. CaMV is often used as a model system as a large amount of information is known about this virus, and it is convenient to use as there are many isolates available that range in pathogenicity (Al-Kaff and Covey, 1995).

CaMV is a member of the Caulimoviridae family, which is the only family of plant viruses with dsDNA genomes. Family Caulimoviridae contains all of the plant viruses that replicate through reverse transcription. All have a circular double-stranded DNA genome with gaps or discontinuities at specific sites, and asymmetric transcription gives rise to RNA, which is longer than the genome (Hull, 2001; Haas *et al.*, 2002). This RNA molecule is both the template for reverse transcription and the mRNA for at least some of the gene products. There are six genera within this family and these fall into two groups, the caulimoviruses and the badnaviruses.

The CaMV virus comprises (T=7) icosahedral particles containing an 8 Kb circular double stranded DNA genome and has been found to encode six potentially eight genes which are closely spaced but with very little overlap except for the possible gene VIII (see **Figure 1.1**; Haas *et al.*, 2002; Hull, 2001; Cheng *et al.*, 1992).



Key:
- Open Reading Frames
- DNA
- Transcripts - 35S RNA, 19S RNA and two spliced forms (SF)

Fig.1.1 Schematic diagram of the CaMV genome showing the seven ORFs and their associated functions. Movement Protein (MP), Aphid Transmission Factor (ATF), Capsid Protein (CP), Transactivator Domain (TAV). Adapted from Ryabova *et al.*, (2004).

The functions or roles of the products produced by these genes have been determined (see **Table 1.1**). CaMV is a member of the pararetrovirus family (Haas *et al.*, 2002). Pararetroviruses have two phases to their replication cycle, a nuclear phase where the viral DNA is transcribed by host DNA-dependent RNA polymerase II: and a cytoplasmic phase where the RNA product of transcription is reverse-transcribed by virus encoded RNA-dependent DNA polymerase or reverse transcriptase (RT) to give DNA.

During replication of CaMV, the dsDNA of the virus particle moves to the cell nucleus, where the overlapping nucleotides at the discontinuities are removed, and the gaps covalently closed to form a fully closed dsDNA (Covey *et al.*, 1990). The covalently closed DNA associates with host histones to form a minichromosome that is used as a template by the host DNA dependent RNA polymerase II to transcribe two RNAs, 19S and 35S. The two polyadenylated RNA species then migrate to the cytoplasm for the next phase of replication. The 19S RNA is translated in large amounts to produce the protein P6 and the 35S mRNA is reverse transcribed to produce a negative DNA strand and once this is complete a positive DNA strand is synthesised. These RNAs are translated by the cellular machinery following two unconventional strategies, ribosomal shunt and termination-reinitiation (reviewed by Rayabova *et al.*, 2002). Virus particles together with viral proteins accumulate in inclusion bodies composed of P6, the product of CaMV ORF VI, which will be discussed later. Virus particles may be taken up by aphids, used to reinfect the nucleus or transported to other cells.

Gene	Product Size (KDa)	Role/Function
I	37	Movement of virus
II	19	Aphid transmission
III	14	Capsid associated protein within virus particle, aphid transmission
IV	57	Precursor of the 42 kDa protein subunit of the icosahedral shell
V	79	Viral reverse transcriptase gene
VI	58	Major protein of viroplasms, significant role in disease induction, symptom expression and in controlling host range. Also functions in trans to activate translation of other viral genes.
VII/VIII	_	Not present in other Caulimoviruses. Function unknown.

Table 1.1 Details of the CaMV open reading frames including protein size and function.

CaMV infects plants of the Cruciferae family, including the Brassicae species and Arabidopsis, often inducing symptoms such as vein clearing, chlorosis and stunting. CaMV infected Arabidopsis seedlings develop mosaics and stunting symptoms, although the character and severity of these symptoms varies, and is dependent on the genetic background of both the host and the CaMV isolate used (Cecchini *et al.*, 1998). A number of CaMV isolates (>20) varying in severity from very mild to very severe are available for studies into the infection process. Cecchini *et al.*, (1998) characterised the pathogenic interactions between variants of CaMV and Arabidopsis, and combinations thought to be useful in molecular genetic studies were identified. When a single Arabidopsis ecotype was infected with a range of CaMV isolates a variety of symptom types were seen. For example, isolate Bari-1 was asymptomatic in ecotype Col-0, and yet caused mild symptoms in ecotype Ler although the levels of virus that accumulated were similar in both ecotypes (Cecchini *et al.*, 1998). Therefore, the symptoms of viral disease in plants must be dependent on specific host loci, which presumably influence symptom expression independently of levels of virus accumulation within the plant (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002). Lee *et al.*, (1996) reported differences in symptom expression in Arabidopsis ecotypes infected with Tobacco ringspot nepovirus (TRSV) that appeared to be independent of virus accumulation. This suggests that it is specific interactions between host and virus gene products that contribute to symptom expression, not necessarily the amount of virus present. Growth conditions and environments can also affect symptom development, with light being an important factor (Cecchini *et al.*, 1998;

Cecchini *et al.*, 2002). Leisner *et al.*, (1992) demonstrated that if the development of early-flowering plants is retarded by growth under sub-optimal conditions, inoculated plants become more susceptible to CaMV and systemic infections more widespread.

A role for the P1 protein has been identified in CaMV cell to cell movement (Thomas *et al.*, 1993; Haas *et al.*, 2002). P1 has been found to be localised to cell walls and specifically to plasmodesmata. It forms tubules through the plasmodesmata, allowing CaMV particles to move from one cell to another (Perbal *et al.*, 1993) There is also significant amino acid homology between the gene I protein and the P30 movement protein of TMV. It has been demonstrated that a central domain of P1 is required for targeting the protein to the cell periphery (Huang *et al.*, 2001), whereas most of the protein, except for the C-terminal is required for tubule formation (Thomas and Maule, 1999). P1 has also been found to contain an RNA-binding domain that partially overlaps the sequence involved in its localization to the cell periphery (Citovsky *et al.*, 1991; Thomas and Maule, 1995).

CaMV is transported systemically through the phloem where it moves with the flow of photoassimilates (Leisner *et al*, 1992; Love *et al.*, 2005). Therefore systemic CaMV infections spread from source leaves to sink leaves. Sink-source relationships change during plant development, as does the pattern of virus

movement. Further evidence for virus movement being dependent on the movement of photoasimilates is that CaMV does not invade leaves that have already gone through the sink to source transition but follows the movement of photoasimilates from source to sink (Leisner *et al.*, 1992). However, in contrast to photoasimilates, CaMV is restricted from developing leaves at a much earlier stage of development (Leisner *et al.*, 1992). Therefore, during development, the region of the plant that is accessible to systemic infection from any given source leaf is progressively reduced. Plants with an early flowering phenotype were reported to show reduced susceptibility to CaMV (Leisner and Howell, 1992) and this is known as 'developmental resistance'. Cecchini *et al.*, (2002) have subsequently shown that mutations that delay flowering enhanced symptom development although virus titres were unaffected. Arabidopsis plants have such a rapid life cycle that even small alterations in environmental conditions can have a profound effect on the apparent susceptibility to systemic CaMV infections.

The product of ORF II P2 is an 18 KDa protein that is involved in the aphid transmission of CaMV but which is dispensable for virus replication (Armour *et al.*, 1983). Several experiments have demonstrated a role for P2 in aphid-mediated transmission of virus particles and P2 is therefore considered to be the aphid transmission factor (ATF). This conclusion was reinforced by the observation that some non-aphid transmissible strains of CaMV contain a mutation that maps to P2, such as Campbell and CM1841 (Haas *et al.*, 2002). P2

is also a major component of the electron-lucent inclusion bodies formed in infected cells; these also contain P3 as well as scattered virions (Drucker *et al.*, 2002). However, P2 is not associated with the viroplasms/inclusion bodies composed of P6, suggesting that after its synthesis P2 is rapidly released and accumulates to form electron-lucent inclusion bodies. P2 self assembles into highly organised paracrystalline filaments in plants (Blanc *et al.*, 1993) via coiled-coil structures involving the C-terminal region (Hebrard *et al.*, 2001).

This region of P2 also interacts with CaMV P3 protein (Leh *et al.*, 1999), so that the formation of P2-P2 and P2-P3 complexes which must somehow be regulated during the viral cycle, play an essential role in the interaction between virions and the aphid stylet. P3 is the 15KDa product of ORF III and has been shown to be closely associated with purified CaMV particles (Dautel *et al.*, 1994). It is associated with the capsid but no structural role has been found. An 11 KDa product resulting from the cleavage of P3 at its C-terminus by a cysteine proteinase is frequently detected when extracts from CaMV infected plants are incubated (Guidasci *et al.*, 1992). P3 has also been shown to play a pivotal role in the formation of a CaMV complex transmissible by aphids (Leh *et al.*, 2001).

The product of ORF IV protein P4, (approximately 56 KDa), is the precursor of the capsid proteins (CP) and corresponds to the gag protein found in retroviruses (Haas *et al.*, 2002). The acidic N- and C-terminals are removed post translationally by the viral aspartic proteinase encoded by ORF V, and by cellular proteases to produce a set of polypeptides ranging is size from 37 to 44

KD (Torruella *et al.*, 1989). These polypeptides possess a zinc finger motif at their C-terminus, which is conserved in all pararetroviruses and in the nucleocapsid proteins of retroviruses. This zinc finger motif is of particular importance, since mutations abolish not only the interaction with RNA but also CaMV infectivity (Guerra-Peraza *et al.*, 2000). Close to their N-terminus the capsid proteins possess a nuclear localization signal; (NLS) which is exposed on the surface of mature virions. Mutations in this region can completely abolish infectivity (Leclerc *et al.*, 1999). Karises *et al.*, (2002) have shown that the acidic N-terminus inhibits nuclear targeting, making it likely that this inhibition acts as a control making sure that the CP precursor is used for virus assembly in the cytoplasm, and that only mature virions reach the nuclear envelope.

The ORF V encodes a polypeptide (P5), of 78 KDa in size. Homology between ORF V of CaMV and the pol gene products of various animal retroviruses indicates that P5 is involved in the replication of CaMV DNA by reverse transcription (Hass *et al.*, 2002). P5 contains an aspartic proteinase and a reverse transcriptase domain in the N-terminal region. However unlike the pol gene from retroviruses it does not contain an integrase motif. The proteinase, 18 KDa is released from P5 by self-cleavage (Torruella *et al.*, 1989). The reverse transcriptase uses tRNA as a primer for the synthesis of (-) DNA strand from the pre genomic RNA. RNAse H digests the RNA template except for a few purine rich sequences, which remain, annealed to the (-) DNA strand and then act as primers for the synthesis of the (+) DNA strand (Haas *et al.*, 2002).

ORF VI encodes a multifunctional 60-66 KD polypeptide (P6) that is essential for CaMV infection. At a cellular level, a prominent cytopathological effect of CaMV infection is the appearance of viral inclusion bodies or viroplasms in the cytoplasm of infected cells (Covey and Hull, 1981). The major inclusion body matrix protein is P6, translated from the 19S RNA. The primary role that has been identified for P6 is modification of the host translation machinery to facilitate the translation of the polycistronic CaMV 35S RNA (Rothnie *et al.*, 1994; Park *et al.*, 2001). This function has been designated the translational transactivator (TAV) function (Bonneville *et al.*, 1989; Scholthof *et al.*, 1992), which is mediated by physical interactions between an internal region, including the minimal sequence of P6 required for transactivation, the initiation factor eIF3 (Park *et al.*, 2001) and the ribosomal proteins L13 (Bureau *et al.*, 2004), L18 (Leh *et al.*, 2000), and L24 (Park *et al.*, 2001). It has since shown that as well as being required for the expression of other CaMV genes, P6 is also involved in host range and symptom determination (Daubert *et al.*, 1984; Baughman *et al.*, 1988; Agma *et al.*, 2002). Construction of recombinants between CaMV variants showing different pathological characters led to the conclusion that the gene VI region contains pathogenic determinants, including those that regulate host specificity and symptom type and severity (Wintermantel *et al.*, 1993; Daubert *et al.*, 1990). *In vitro* mutagenesis of P6 suggests that the pathogenicity determinants map to the N-terminal region and may be distant from the TAV and interaction domains (Kobayashi and Hohn, 2003). Further evidence implicating gene VI as an important CaMV pathogenic determinant comes from experiments

in which P6 has been expressed as a transgene in plants in the absence of virus infection.

Expression of P6 in both nonhost, *Nicotiana tabacum*, and host plants, Arabidopsis leads to the development of symptom-like leaf mosaics, mottling, chlorosis and necrotic spots (Baughman *et al.*, 1988; Goldberg *et al.*, 1991; Cecchini *et al.*, 1997; Zijlstra and Hohn, 1996) and in Arabidopsis P6 transgenics, cells contained empty inclusion bodies, characteristic of infected plants (Cecchini *et al.*, 1997). The symptom character was dependent on the level of P6 expression, and on the isolate from which the P6 originated (Cecchini *et al.*, 1997). Geri *et al.* (1999) performed differential display polymerase chain reactions to identify changes in gene expression in P6 transgenic Arabidopsis. They identified changes in the abundance of more than 30 host genes, (either up or down- regulated), and they noted that the altered gene expression patterns were broadly similar to those observed in CaMV-infected Arabidopsis.

Plant Hormones and Responses to Virus Infection (1.5)

Plant hormones are signal molecules, and changes in their concentration and quantity regulate a whole range of developmental processes, many of which involve interactions with environmental factors. Virus infection can result in the alteration of physiological, biochemical, and metabolic processes within the plant (Fraser, 1987). The alterations in plant growth can be regarded as cytopathic effects of virus infection (Wenham and Fraser, 1990), which may be

manifested in alterations of plant hormone metabolism. During virus infection, the symptoms that develop may include those that reflect changes in the biosynthesis and metabolism of plant hormones (reviewed by Pennazio and Roggero, 1996). Early investigations into the effects of virus infection on plant hormones showed varied results, but pointed towards a role in plant pathogen interactions. However, since the 1970s, little new work has been done on the role of classical plant hormones such as gibberellic acid (GA), abscisic acid (ABA), auxin and cytokinin in virus infection. Past investigations that tried to establish the role these hormones play in virus infection relied mainly on techniques such as bioassays and local lesion assays, where crude infected sap from hormone-treated material was inoculated onto a secondary host and local lesion production recorded (Jameson and Clarke, 2002; Fraser and Whenham, 1987). Attempts were made to measure hormone levels in infected plants using techniques that were only semi-quantitative and often gave rise to inaccurate and inconsistent results. For example, Rajagopal (1977) showed a decrease in abscisic acid (ABA) concentration in TMV-infected tobacco in the early stages of virus infection whereas Keller and Luttge (1991) showed that infected plants had a higher ABA content (Jameson, 2002). Only a limited number of studies of GA content following virus infection have been carried out and the majority of these reported a decrease in GA activity in virus-infected tissues. Ben-Tal and Marco (1980) reported a significant reduction in GA concentration in cucumber mosaic virus (CMV) - infected cucumber. More recently Zhang *et al.*, (1997) used a GA based ELISA to confirm earlier studies reporting a decrease in GAs in diseased compared with healthy tissue. Kuriger and Agrios (1977) demonstrated a decline

in cytokinin activity in root exudates of cowpea infected with tobacco ringspot virus (TRSV), whereas Sziraki *et al.*, (1980) reported that systemic infection by CMV caused an increase in cytokinin content in leaves of tobacco (Pennazio and Roggero, 1996). The inconsistency of these results has made it impossible to define the role of these hormones or even determine if they are involved at all.

More specifically, the plant hormones ethylene, jasmonic acid (JA) and salicylic acid (SA) have been shown to be important in plant defence responses against a variety of plant pathogens (for review see Dong, 1998; Raymond and Farmer, 1998) and a number of investigations have been carried out to establish the role of these hormones in controlling plant defence mechanisms during virus infection (see for example Knoester *et al.*, 2001; Penninckx *et al.*, 1998; Niki *et al.*, 1998; O'Donnell *et al.*, 2003; Delaney *et al.* 1994).

Ethylene (1.5.1)

Ethylene is an endogenous plant growth regulator that affects many aspects of plant growth and development (reviewed by Stepanova and Ecker, 2000). Ethylene is among the best-characterised plant hormones and participates in processes such as fruit ripening, inhibition of stem and root elongation, senescence, promotion of seed germination and flowering, and sex determination. Over the past few years understanding of ethylene's role in these processes, and the ethylene-signalling pathway have greatly increased. The major components of the ethylene-signalling pathway have been identified and

characterized through the use of genetic studies in Arabidopsis. This began with the identification of mutants defective in the ethylene triple response. Arabidopsis seedlings grown for three days in the dark under continuous exposure to ethylene exhibit a phenotype that is known as the "triple response" (Ecker, 1995). This phenotype includes thick, short roots and hypocotyls, and an exaggerated curvature of the apical hook. Screens for mutants unable to generate the triple response when treated with ethylene have been used to identify ethylene insensitive mutants and those that show the triple response in the absence of ethylene are either ethylene overproducing or constitutive-signalling mutants (Bleecker *et al.*, 1988).

Over twelve genes have now been implicated in the ethylene signalling pathway and their order of action carefully determined. **Figure 1.2** shows a current model of the ethylene signal transduction pathway. In Arabidopsis a family of integral membrane receptors perceive ethylene. This family consists of at least five members, Ethylene Triple Response 1 (ETR1) and Ethylene Receptor 2 (ETR2), Ethylene Insensitive 4 (EIN4), and Ethylene Response Sensor 1 and 2 (ERS1 and ERS2) (Chang *et al.*, 1993; Hua *et al.*, 1995; Sakai *et al.*, 1998). Ethylene binds to these receptors via a copper cofactor (Rodriguez *et al.*, 1999) and studies have suggested that binding of the hormone inactivates the receptors (Hua and Meyerowitz, 1998). The receptors are predicted to be functionally active histidine-kinases that activate a Raf-like serine/threonine kinase, Constitutive Triple Response 1 (CTR1) which is also a negative regulator of the ethylene pathway and a MAPKK (Keiber *et al.*, 1993). Other components of the

pathway such as, EIN2, EIN3, EIN5 and EIN6 are thought to be positive regulators of the ethylene response (Roman *et al.*, 1995). Epistasis studies have now revealed that ETR1, ETR2 and EIN4 along with the homologs ERS1 and ERS2, work upstream of CTR1, whereas EIN2, EIN3, EIN5 and EIN6 appear to work downstream of CTR1. Ethylene Insensitive 3, EIN3, is believed to work downstream of EIN2 and serve as a transcription factor that, in response to ethylene binds to specific sequences in the promoters of target genes and activates their transcription. Ethylene Response Factor 1 (ERF1) is a positive regulator of ethylene signalling and is the only known target of EIN3 (Solano *et al.*, 1998; Chao *et al.*, 1997). In Arabidopsis EIN3 binds to the GCC-box in the promoters of several ethylene responsive genes (Solano *et al.*, 1998), including some defence related genes. Given the range of responses to ethylene, it seems that some ethylene-related phenotypes result from the activation of some genes and the repression of others

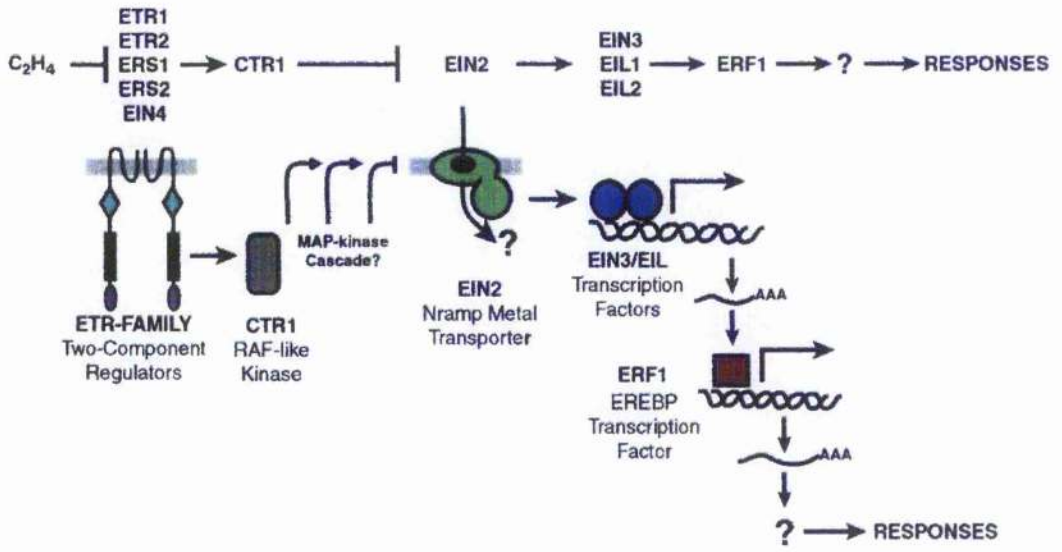


Fig 1.2 Ethylene signalling pathway. Diagram from: Bleecker (2000).

As well as elucidation of the ethylene signalling pathway, it has been shown that ethylene plays a central role in plant disease resistance and symptom development (reviewed by van Loon *et al.*, 2006; Knoester *et al.*, 2001; Thomma *et al.*, 1999; Geraats *et al.*, 2002). Ethylene gas is frequently synthesised during both incompatible and compatible host-pathogen interactions (Johnson and Ecker, 1998) and is thought to be a part of the plant defence mechanism against the spread of pathogens. In the past few years it has been demonstrated that a functional ethylene-signalling pathway is required for resistance against some but not all plant pathogens (Stepanova and Ecker, 2000). This was illustrated by Knoester *et al.*, (1998) who made use of transgenic tobacco plants transformed with a dominant-negative mutant allele (*etr1-1)* of the Arabidopsis ethylene receptor gene *ETR1*. The transgenic plants with a disrupted ethylene response were more susceptible than wild-type plants to normally non-pathogenic soil-borne *Pythium spp*, whereas their level of resistance to TMV was unaffected.

It has also been shown that ethylene production appears to be increased by virus infection and that it has been associated with the development of necrotic or chlorotic lesions, correlated with the suppression of hypocotyl elongation or associated with other growth abnormalities. The majority of the work has focused on systems in which a hypersensitive response occurs but Chaudhry *et al.*, (1998) specifically showed enhanced ethylene production in leaves of tobacco systemically infected with CMV. Love *et al.*, (2005) reported that CaMV infection stimulated the expression of ethylene responsive markers in Arabidopsis and have shown that two ethylene insensitive

mutants show reduced susceptibility to CaMV infection. The use of biosynthetic inhibitors, ethylene action inhibitors, or hypobaric conditions have all been shown to relieve virus-induced symptoms, including reducing lesion size (Ohtsubo *et al.*, 1999) delaying chlorotic lesion development (Marco and Levy, 1979), and increasing hypocotyl elongation and preventing virus-induced epinasty, all of which support causative role of ethylene in the development of these symptoms.

Although it is evident that ethylene plays a specific role in plant disease resistance, its effect on plant-pathogen interactions is pleiotropic and depends on the host and pathogen species and conditions tested. For example, ethylene overproduction in tobacco resulted in enhanced lesion formation upon TMV infection (Ohtsubo *et al.*, 1999). However, ethylene insensitivity had no effect on the hypersensitive response or the overall resistance of Arabidopsis plants to Turnip crinkle virus (TCV) infection (Kachroo *et al.*, 2000). Takahashi *et al.*, (2002) demonstrated through the use of Arabidopsis plants carrying the RCY1 resistance gene but defective in SA, JA and ethylene signalling that there is a requirement for ethylene and SA signalling in mounting a resistance response to CMV-Y.

Jasmonic Acid (1.5.2)

Jasmonic acid is a plant growth regulator derived from oxygenated fatty acids via the octadecanoid pathway and characterised by a pentacyclic ring structure

(reviewed by Schaller *et al.*, 2005). Regulated changes in the relative levels of this hormone and other forms of jasmonates contribute to the control of metabolic, developmental and defensive processes in plants. Jasmonates have been shown to be involved in fruit ripening, production of viable pollen, root growth, response to wounding and abiotic stress as well as defence against insects and pathogens. Increases in JA in response to pathogen or insect attack occur both locally and systemically (Schaller *et al.*, 2005). Reduced Potato virus M (PMV) content in potato meristems was correlated to increased jasmonic acid levels (Ravnikar *et al.*, 1990). Measurement of JA with radioimmunoassay showed that JA and its amino acid conjugates occur at different concentrations in virus-free and in PVM infected potato plants. They found that JA, in addition to a previously reported effect on senescence, increases the number of meristems which developed in plants *in vitro* and increases the proportion of mature virus-free potato plants (*Solanum tuberosum* L. cv. Vesna) completely infected with PMV.

A role for JA in plant defence was first shown by Farmer and Ryan (1990) who demonstrated the induction of proteinase inhibitors by methyl jasmonate (MeJA) and JA as part of the defence response against herbivorous insects. JA is now considered to be one of the key compounds in plant defence reactions to both biotic and abiotic stimuli. Jasmonates have been shown to be active inducers of antimicrobial phytoalexins (Gundlach *et al.*, 1992) and to induce defence gene expression for enhanced resistance against insect predators and pathogens (Blee *et al.*, 2002; reviewed by Reymond and Farmer, 1998). The regulation of insect-

and pathogen-defence-gene expression is affected in mutants that fail to respond to JA like *coi1*, *jar1* and *jin1* mutants in Arabidopsis (Berger *et al.*, 1996; Staswick *et al.*, 2002; Lorenzo *et al.*, 2004) or the *jai1* mutant in tomato (Li *et al.*, 2004). It has been demonstrated that the production of jasmonates leads to the induction of many defence related genes including PDF1.2 a plant defensin (Pennickx *et al.*, 1998).

Salicylic Acid (1.5.3)

Salicylic acid (SA) is a central signalling intermediate in plant defence (Dempsey *et al.*, 1999). SA accumulates in plant tissues following a hypersensitive response (HR), a resistance response most often characterised by programmed host cell death at the point of attempted entry by the pathogen (Heath, 2000). The occurrence of HR is usually associated with an enhancement in resistance to a broad spectrum of pathogens throughout the plant called systemic acquired resistance (SAR; Dempsey *et al.*, 1999). This state of enhanced resistance can also be induced by treatment of plants that have not undergone an HR with SA or its synthetic analogues (Dempsey *et al.*, 1999; Murphy *et al.*, 2001). SA is known to induce expression of many of the pathogenesis-related (PR) proteins involved in plant defence as well as limiting the replication and movement of viruses throughout the plant (Murphy and Carr, 2002; Hooft van Huijsduijnen *et al.*, 1986; Naylor *et al.*, 1998). In recent years another layer of SA-regulated defence has been identified which is based upon RNA silencing although this link is not fully established (reviewed by Singh *et al.*, 2004). RNA silencing is a sequence specific RNA degradation process,

affecting all highly homologous sequences in which foreign, over-expressed or aberrant RNA molecules are targeted for destruction in a sequence specific manner (Baulcombe, 2001; Voinnet, 2001). This mechanism can contribute to resistance to viruses, and it has been demonstrated that a number of plant and animal viruses encode silencing suppressor proteins (Li *et al.*, 2002; Vance and Vaucheret, 2001). The possibility that a fuctional connection exists between SA-induced resistance to viruses and RNA silencing came from the discovery that a tobacco gene encoding an RNA-dependent RNA polymerase (RdRp) was induced by SA (Xie *et al.*, 2001). This is significant as RdRps from host plants can play important roles in the induction of RNA silencing (Ahlquist, 2002). Also the CMV protein 2b affects both RNA-silencing and SA-dependent defence responses (Ji and Ding, 2001)

Reactive oxygen species (ROS) are often produced during the early stages of a plant resistance response (Love *et al.*, 2005; Wojtaszek, 1997; Lamb and Dixon, 1997; Alvarez *et al.*, 1998). Production of ROS can significantly alter the redox balance within cells responding to the virus and it has become widely accepted that cellular redox or changes in redox state can influence the activity of protein molecules that participate in defence signalling, e.g. PR gene induction (Pastori *et al.*, 2003, Koch *et al.*, 1998). One of the ways in which a plant can regulate ROS generation while maintaining electron flow into the respiratory chain is through the activation of the Alternative Oxidase (AOX; Maxwell *et al.*, 1999).

It is clear that SA plays an essential role in plant resistance to virus infection but it will not be discussed further as it is not directly relevant to the main aims of the thesis.

Integration of Viral Defence pathways (1.5.4)

There is a growing body of literature that reports that JA, SA and ethylene defence signalling pathways do not function independently (Reymond and Farmer, 1998; Dong, 1998). It is thought that they are involved in a complex signalling network in which the different pathways influence each other through both positive and negative regulatory interactions.

Several studies have shown positive interactions between JA and ethylene signalling pathways (Reymond and Farmer, 1998; Dong, 1998). Both JA and ethylene signalling are required for the expression of the defence related gene *PDF1.2* in response to infection by *Alternaria brassicicola*, and for the expression of *PDF1.2*, *HEL* and *CHIB* in response to treatment with *Erwinia carotovora* culture filtrates (Penninckx *et al*., 1996). Furthermore, when both are applied exogenously to plant tissue, JA and ethylene appear to function synergistically to induce *PDF1.2*, *HEL* and *CHIB* in Arabidopsis.

There is a limited amount of data that suggests both positive and negative regulatory interactions between ethylene and SA. For example, SA and ethylene may function together to co-ordinately induce several defence related genes (Manners *et al*., 2000), whilst the basal level of PR-1 mRNA appears to be

It is clear that SA plays an essential role in plant resistance to virus infection but it will not be discussed further as it is not directly relevant to the main aims of the thesis.

Integration of Viral Defence pathways (1.5.4)

There is a growing body of literature that reports that JA, SA and ethylene defence signalling pathways do not function independently (Reymond and Farmer, 1998; Dong, 1998). It is thought that they are involved in a complex signalling network in which the different pathways influence each other through both positive and negative regulatory interactions.

Several studies have shown positive interactions between JA and ethylene signalling pathways (Reymond and Farmer, 1998; Dong, 1998). Both JA and ethylene signalling are required for the expression of the defence related gene *PDF1.2* in response to infection by *Alternaria brassicicola*, and for the expression of *PDF1.2*, *HEL* and *CHIB* in response to treatment with *Erwinia carotovora* culture filtrates (Penninckx *et al.*, 1996). Furthermore, when both are applied exogenously to plant tissue, JA and ethylene appear to function synergistically to induce *PDF1.2*, *HEL* and *CHIB* in Arabidopsis.

There is a limited amount of data that suggests both positive and negative regulatory interactions between ethylene and SA. For example, SA and ethylene may function together to co-ordinately induce several defence related genes (Manners *et al.*, 2000), whilst the basal level of PR-1 mRNA appears to be

significantly elevated in *ein2* mutant plants (Lawton *et al.*, 1994). This contradictory evidence may reflect the complexity of the interactions between ethylene and SA signalling pathways.

Interactions between SA and JA also appear to be highly complex, and as is the case with ethylene and SA there is evidence for both positive and negative regulatory interaction between these pathways (Doares *et al.*, 1995a; Doares *et al.*, 1995b). Several studies have provided evidence for an antagonistic effect of SA on JA signalling in Arabidopsis. The *eds4* and *pad4* mutants, which are impaired in SA accumulation, exhibit enhanced responses to inducers of JA-dependent gene expression (Gupta *et al.*, 2000). There is also growing evidence for an antagonistic effect of JA on SA signalling. Studies in tobacco have shown that JA inhibits the expression of SA dependent genes (Ohashi *et al.*, 1998).

Cross talk between signalling pathways is believed to be an important factor in allowing plants to respond effectively to a wide range of pathogens and allowing them to fine tune there response and to ensure that the right combination of defences are deployed against specific pathogens.

Auxin (1.6)

The relationship between auxin and virus diseases was first investigated by Grieve (1936) who studied the role of IAA in stunting in tomato plants infected with Tomato spotted wilt virus (TSWV). Grieve used the techniques employed by Went (1926) to compare the effects of diseased and healthy sap upon the inward curvature induced by auxin on split pea stems. In four experiments, such curvatures were observed upon the stems placed in healthy sap with auxin but were absent from those treated with virus infected sap and the hormone. This seemed to show an inactivation of the growth regulator by the virus, but a further number of similar experiments did not indicate any difference between the two kinds of treatment. Another more conclusive method of study involved the injection of various amounts of auxin into healthy, and into diseased tomato plants. Adventitious roots appeared upon the stem in the majority of healthy plants, but did not form upon the diseased tomatoes, unless the plant appeared to have thrown off the disease. Later, Baldacci (1953) suggested that IAA was liberated in virus-infected potato and that it was responsible for the resultant symptoms and distortions (Pennazio and Roggero, 1996). Most reports to date indicate a reduction in auxin activity and/or concentration in diseased plants, generally associated with stunting (Fraser and Whenham, 1982; Pennazio and Roggero, 1996). Smith *et al.*, (1968) found reduced auxin activity in bean, sugarbeet and tomato plants when infected with beet curly top geminivirus (BCTV). However, a substantial increase in auxin activity has sometimes been observed in some cases showing severe symptoms (Jameson *et al.*, 2002). In

both TSW- and TMV- infected tomato plants, Jones (1956) found auxin activities higher than in healthy control plants, although the former were visibly stunted (Fraser and Whenham, 1982).

Other evidence suggests that wound, pathogen and auxin perception may share conserved signal transduction pathways that cross-talk at some point. For example expression of a pathogen-induced gene can be mimicked by auxin insensitivity. The *CEVI-1* gene is found in tomato plants and its expression is up-regulated during the course of compatible viral infections with citrus exocortis viroid (CEV; Gadea *et al.*, 1996) but not in incompatible interactions or by the application of signalling molecules such as SA, ethylene or JA (Mayda *et al.*, 2000). The homologous Arabidopsis mutants, called *detachment* (*dth*) mutants that are deregulated in the control of expression of transgenic *CEVI-1* have been identified. From initial analysis of two of these mutants, *dth2* and *dth23*, both the activation of *CEVI-1* gene expression and an auxin-resistant phenotype occurred (Mayda *et al.*, 2000). These results seem to imply that during systemic infections with viruses, auxin homeostasis is one of the components participating in the regulation of the overall defence response. It has also been observed that elicitor induced expression of some of the defence-related genes in tobacco protoplasts are negatively regulated by auxin (Jouanneau *et al.*, 1991). In tobacco, preparation of protoplasts without auxin appeared sufficient to induce a spontaneous defence reaction and it was also found that the accumulation of PR proteins was maximal when tobacco and soybean protoplasts were prepared and further cultured in the absence of auxin (Jouanneau *et al.*, 1991).

More recently, Navarro *et al.*, (2005) have shown that a microRNA, which negatively regulates messenger RNAs for the F-box auxin receptors *TIR1*, *AFB2* and *AFB3* and subsequently represses auxin signalling, can lead to resistance to *Pseuidomonas syringae*. This implicates auxin in disease susceptibility and miRNA-mediated suppression of auxin signalling in resistance.

Unfortunately, the mechanisms leading to changes in auxin level in virus-infected plants are still unknown. Therefore, no clear correlation between changes in auxin levels and distribution and impaired plant growth and development has been found. To do so more accurate quantitation of hormone levels and distribution along with molecular genetic approaches are needed.

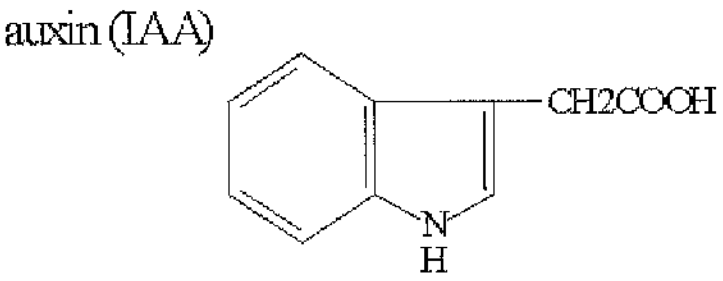


Fig 1.3 Chemical structure of Indole-3-acetic acid (IAA)

The main form of auxin in higher plants is indole-3-acetic acid (IAA), which is found both free and as conjugates within the plant (see **Figure 1.3**). Auxin is thought to regulate a variety of responses in plants including rates of cell expansion, rates of cell division and establishment and maintenance of pattern formation during growth and development (Hobbie *et al.*, 1998). The role of auxin in cell elongation has been demonstrated by studies on hypocotyl elongation. For example, when auxin is added to isolated stem segments and coleoptiles it is able to induce elongation (Collet *et al.*, 2000). A role for auxin in elaborating the embryonic axis has been suggested as polarity defects can be induced in embryos by blocking normal auxin movement (Hadfi *et al.*, 1998).

Auxin has been found to be unique among plant hormones as it has its own dedicated transport system. Auxin is transported in a polar fashion from the shoot apex down the stem towards the roots, largely through cells associated with the vasculature. This transport system has been termed Polar Auxin Transport (PAT) and has been found to occur in a basipetal (apex to base) direction within the aerial tissues of plants and in two distinct streams within root tissues, both basipetal and acropetal (base to tip) directions. Rubery and Sheldrake (1974) and Raven (1975) proposed within their chemiosmotic hypothesis that the polarity of IAA transport reflects the asymmetric subcellular distribution of auxin efflux and possibly influx carrier proteins within specialised transport cells (Rubery and Sheldrake, 1974; Raven, 1975).

Knowledge about PAT has been advanced through the identification of Arabidopsis mutants that have defective auxin responses. The use of suitable inhibitors of auxin response has been very useful in identification of the carrier complexes that mediate auxin transport (Bennett *et al.*, 1998). The majority of this work has focused on the efflux carrier as work on the influx carrier has been held back by the lack of suitable inhibitors. Morris *et al.*, (1991) suggested that the efflux carrier consists of at least three polypeptides, which include a transmembrane carrier protein, NPA binding protein and a third labile component.

The *PIN1* (PIN Formed 1) Arabidopsis gene has been proposed to encode a component of the efflux carrier complex based first on its mutant phenotype, which can be phenocopied by culturing wild-type seedlings in the presence of the efflux inhibitor NPA, and second, on the significantly reduced rates of polar auxin transport within mutant inflorescence tissues (Okada *et al.*, 1991). *PIN1* encodes a membrane-localised protein, prompting the suggestion that it may represent the transmembrane component of the efflux carrier. *AtPIN1* shows similarity to some bacterial transporters and it has been shown that disruptions in this gene affect PAT, patterning in the embryo and vascular development (Swarup *et al.*, 2001). Petrasek *et al.*, (2006) have shown recently that PINs mediate auxin efflux from mammalian and yeast cells without needing additional plant-specific factors. Conditional gain-of-function alleles and quantitative measurements of auxin accumulation in Arabidopsis and tobacco cultured cells have revealed that the action of PINs in auxin efflux is rate-limiting, specific to

auxins, and sensitive to auxin transport inhibitors. Therefore suggesting a direct involvement of PINs in catalyzing cellular auxin efflux.

The most likely candidates for additional auxin transport proteins are the plant orthologs of mammalian multidrug resistance/P-glycoproteins (MDR/PGPs). In mammalian systems, MDR/PGPs function in ATP-dependent movement of hydrophobic substrates, and there is evidence that a subset of the 21-member PGP-type ATP-binding cassette ABC transporter family play a similar role in auxin efflux in plants. Defective PGP1 and PGP19 result in decreased auxin transport and reduced growth phenotypes in Arabidopsis, maize and sorghum. PGP1 has been shown to mediate the efflux of auxin from Arabidopsis protoplasts and heterologous systems such as yeast and HeLa cells (Geisler *et al.*, 2005) It has also been shown that PGPs interact with PIN and that although they characterise coordinated, independent auxin transport they also function interactively in a tissue-specific manner (Blakeslee *et al.*, 2005). It has been shown that PGP1 and PGP19 colocalize with PIN1 in the shoot apex in Arabidopsis and with PIN1 and PIN2 in root tissues. Specific PGP–PIN interactions were seen in yeast two-hybrid and coimmunoprecipitation assays and PIN–PGP interactions appeared to enhance transport activity and, to a greater extent, substrate/inhibitor specificities when coexpressed in heterologous systems. It is likely that PGPs mediate the ATP-dependent transport of IAA in conjunction with PIN proteins, which offer directionality and substrate specificity.

Another component of the auxin efflux carrier was thought to be the product of the *TIR3* (Transport Inhibitor Response 3) gene. Ruegger *et al.* (1997) described the *tir3* mutant, which exhibits a reduced rate of polar auxin transport. Studies also observed that the mutant had significantly reduced levels of NPA binding which led to the suggestion that the *TIR3* gene may encode or regulate the activity of the NPA binding protein associated with the efflux carrier (Ruegger *et al.*, 1997). However, Gil *et al.*, (2001) have demonstrated that *tir3-1* is allelic to the *doc1-1* mutation. The *DOC1* (Dark Over expressing *CAB* 1) gene is a putative component of the light signal transduction pathways (Li *et al.*, 1994). The *DOC1* locus has been recently renamed as *BIG* (Gil *et al.*, 2001) and is predicted to encode a large protein (560 KD) with significant similarity to the *Drosophila* protein Calossin/Pushover. Calossin is believed to be involved in the control of synaptic transmission at the neuromuscular junction, in a process that requires the synaptic vesicle cycle (Xu *et al.*, 1998). Like synaptic transmission, the asymmetric distribution of auxin efflux carriers at the plasma membrane depends on targeted vesicle transport (Steinmann *et al.*, 1999) and recent work has shown that *BIG* is essential for proper positioning of the auxin efflux carrier, *PIN1*, at the plasma membrane (Paciorek *et al.*, 2005). Therefore *TIR3* is essential for either the synthesis, localization, or the function of the NPA binding site (Gil *et al.*, 2001) but is not a component of the carrier itself.

The influx carrier was first described by Rubery and co-workers who observed a saturatable component for auxin uptake within suspension cell cultures and stem segments (Rubery and Sheldrake, 1974; Davies and Sheldrake, 1978). More recently, molecular genetic studies in Arabidopsis (Swarup *et al.*, 2001) have identified putative auxin influx carrier components encoded by the *AUX1* gene sequence. The Arabidopsis *AUX1* gene was isolated using a gene tagging approach and found to encode a highly hydrophobic polypeptide featuring up to 11 membrane-spanning domains. Colinearity between *AUX1* and a family of sequences that encode plant amino acid permeases (Frommer *et al.*, 1993) was found, suggesting that they may share a common ancestry and domain structure. Plant amino acid permeases facilitate the uptake of amino acids into plant cells; Bennett *et al.* (1996) have suggested that *AUX1* may therefore perform a similar transport role, facilitating the movement of the amino acid-like signalling molecule, IAA.

Carrier mediated uptake requires a proton motive force because the influx carrier functions electrogenically, transporting one proton with every protonated IAA molecule (see **Figure 1.4**). As a weak acid, IAA is rapidly protonated in the apoplastic space, then taken up by the plant cell either by diffusion or via the auxin influx carrier. Once within the plant cytosol (pH 7.0) the dissociated IAA^- is removed by a distinct auxin efflux carrier (Delbarre *et al.*, 1996). There is also growing evidence in Arabidopsis for a non-PAT mode of auxin redistribution from auxin source tissues such as young leaves to the roots via the phloem

(Swarup *et al*., 2001). It has been found from Mass Spectrometry (MS) measurements that a significant proportion of root apical IAA is unloaded by AUX1 from a phloem source (Swarup *et al*., 2001). AUX1 localises to the protophloem cell poles, therefore revealing a novel apoplastic transport pathway for delivering phloem borne IAA to the root apex.

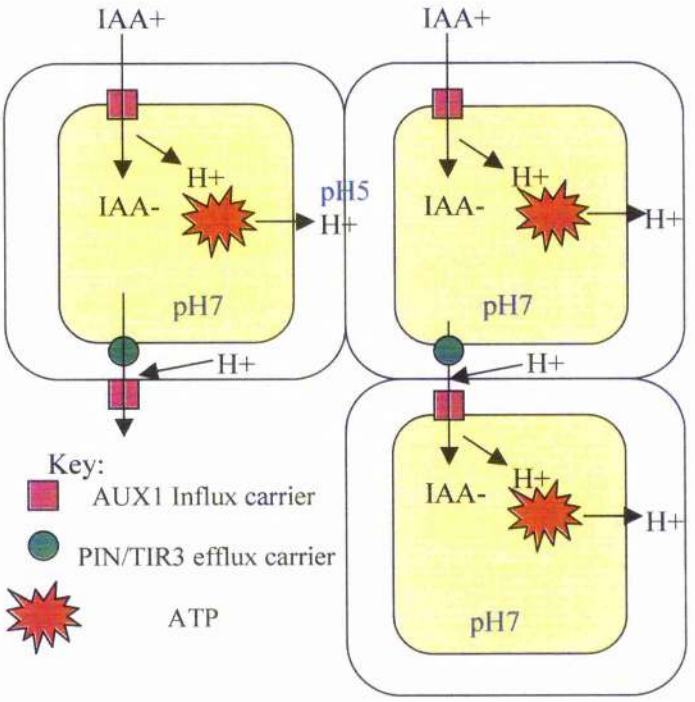


Fig 1.4 Model of cellular auxin transport. IAA moves into cells via the *AUX1* influx carrier and exits through the efflux carrier. The efflux carrier consists of three components including *PIN1* and *TIR3*.

Other work on the auxin-signalling pathway has demonstrated that in wild-type Arabidopsis plants auxin sensitivity depends on the correct functioning of targeted protein degradation (Gray *et al.*, 2000; del Pozo and Estelle, 2000). Wild-type auxin signalling depends on the function of a ubiquitin ligase complex of the SCF type (see **Figure 1.5**). SCF complexes are found in a range of eukaryotes and consist of an SKP1 homologue, a Cullin/CDC53 homologue, an F-box protein and an RBX1/ROC1 homologue (Leyser, 2001). The F-box protein determines which proteins are degraded in the proteasome and consequently SCF complexes are considered to be the hub of the degradative machinery. Arabidopsis mutants that are defective in several components of this system have been recovered and found to share a variety of morphological phenotypes that reflect their reduced sensitivity to auxin. The first gene to be identified in this way was Auxin Resistant 1 (*AXR1*), which encodes a protein with similarity to the amino terminal half of ubiquitin activating enzyme E1. E1 catalyses the first step in the pathway leading to the conjugation of ubiquitin (UBQ) to specific target proteins. The UBQ is then passed to ubiquitin conjugating enzyme E2 and from there to target proteins, often requiring the action of a ubiquitin-protein ligase E3. It is the E3 enzyme that confers target specificity, and in the case of auxin regulated protein degradation is encoded by the *TIR1* gene. *TIR1* encodes an F-box protein similar to the yeast proteins Cdc4p and Grr1p and mutations in this gene result in an auxin resistant phenotype quantitatively similar to that of *axr1* but less severe (Ruegger *et al.*, 1997; Leyser, 2001). *TIR1* interacts with plant orthologs of Skp1 (*ASK1* and

ASK2) and a cullin protein to form SCF^{TIR1}. The proteins targeted for degradation by this SCF^{TIR1} complex are members of the AUX/IAA family of transcriptional repressor proteins, of which there are 29 in Arabidopsis. AUX/IAAs can dimerize with and repress the activity of, transcriptional activators of the auxin response factor (ARF) family of DNA binding proteins. Thus the degradation of AUX/IAAs leads to the derepression of ARF-mediated transcription. Therefore the interaction between the AUX/IAA and the SCF^{TIR1} complex is central to auxin biology. More recently, results have shown that auxin binds directly to the SCF^{TIR1} complex, indicating that TIR1 is the auxin receptor for this response.

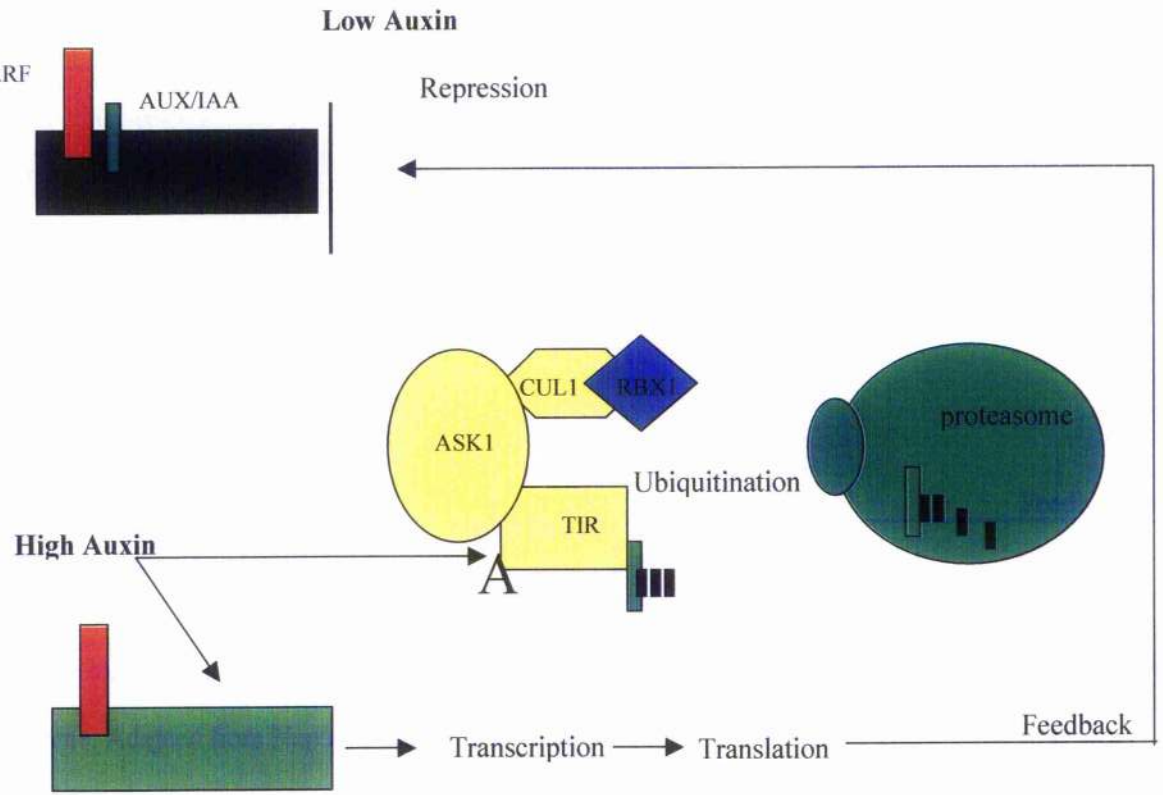


Fig 1.5 Auxin regulated gene expression through targeted degradation of the AUX/IAA transcription factors by SCF-type ubiquitination and degradation in the proteasome. Adapted from Napier, (2005).

Further upstream, the site of auxin perception is still being debated (Hertel, 1995). The most likely candidate for an auxin receptor is Auxin Binding Protein 1 (ABP1). *ABP1* was first identified because of its ability to bind specifically to auxin and there is good evidence to suggest that it regulates downstream auxin responses (Hobbie *et al.*, 1998; Leyser, 2001). Over expression of *ABP1* in transgenic cell lines and plants can alter auxin sensitivity in a variety of responses, including cell expansion and guard cell potassium currents. More recently an insertion in the *ABP1* gene from Arabidopsis has been identified, allowing analysis of the null mutant phenotype. Plants that are homozygous for this insertion die in the early globular stage of embryogenesis, clearly demonstrating the importance of this gene. However, it is thought that there are multiple sites of auxin perception and so it is possible that *ABP1* is not the only receptor. Other possible receptors are the auxin efflux carriers themselves and as mentioned previously TIR1 (Napier, 2005). TIR1 functions as the F-box component of the SCF complex. Which regulates targeted protein degradation Work from two separate groups, has shown that TIR1 binds auxin directly and therefore the auxin receptor for this degradation pathway (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005). It has been demonstrated that TIR1 after purification retains the ability to interact with Aux/IAA substrates in an auxin dependent manner, that IAA binding correlates with TIR1 abundance and TIR1 produced in the Xenopus oocyte expression system exhibits saturable, high affinity auxin binding. These studies provide the receptors for auxin-mediated

gene activation and, as such, receptors for most of the large array of responses controlled by auxin. However, there are also responses that TIR1 signalling cannot account for. It is likely that rapid responses and those responses initiated at the cell surface are not TIR1 mediated. It is possible that these are mediated by ABP1.

Integration of the Auxin Signalling Pathway (1.7)

Interactions between signal transduction pathways furnish a rapid and efficient tuning mechanism for optimising non-cognitive behaviour in response to various combinations of stimuli. These connections between the pathways are termed cross-talk (Genoud and Metraux, 1999). Shared signal transduction pathways might mean that fewer elements are needed for overall hormone signalling.

As a non-cell autonomous signalling mechanism, polar auxin transport is a frequent target for other signalling pathways to regulate plant development. Mutant screens that were originally designed to isolate lesions within auxin signal transduction very often identified mutants with altered responses to multiple hormones (Ephritikhine *et al.*, 1999; Tiryaki and Stawsick, 2002). For example mutants screened for ability to grow on inhibitory concentrations of ethylene were subsequently identified as alleles of *aux1* (Pickett *et al.*, 1990). *tir3-1* mutant which was originally identified from a screen for plants with altered responses to auxin transport inhibitors has now been demonstrated to be the *doc1-1* mutant and has roles in vesicle trafficking and in the light signal

transduction pathways (Gil *et al.*, 2001). This highlights the occurrence of "cross-talk" between auxin and other signalling pathways (Swarup *et al.*, 2001).

Auxin appears to interact with many of the other plant hormones. Of these interactions, the cross-talk between auxin and ethylene and auxin and JA are probably the most significant in terms of response to virus infection, since both, ethylene and JA have known roles in plant defence. Auxin and ethylene co-ordinately regulate several developmental programs in plants. For example, both are required to regulate apical hook formation (Raz and Ecker, 1999; Lehman *et al*, 1996), root hair differentiation, root hair elongation (Pitts *et al*, 1998), root hair growth, and hypocotyl phototropism (Harper *et al*, 2000). They have been described to interact at the level of ethylene biosynthesis (Swarup *et al.*, 2002). Given that auxin stimulates the production of ethylene, it is often unclear whether developmental effects attributed to auxin are due to auxin, ethylene, or synergistic interaction between both classes of phytohormones. Auxin and JA cross-talk studies appear to show that jasmonic acid inhibits auxin-regulated elongation in etiolated oat coleoptiles (Swarup *et al.*, 2002).

Other hormones whose signalling pathways appear to interact with auxin include ABA, cytokinin and GA. For example, ABA and auxin have been observed to interact antagonistically to regulate stomatal aperture (Eckert and Kaldenhoff, 2000). Auxin and GA have been described to co-ordinately regulate several developmental programs in plants including pea stem elongation (Ross and O'Neill, 2001) and parthenocarpy (Ikeda *et al.*, 1999). It appears that auxin

and GA regulate each other's biosynthesis (Ross *et al.*, 2000). Finally, auxin and cytokinin appear to co-ordinately regulate shoot cell proliferation by controlling the expression of the interacting cell cycle regulating components, cdc2 and cycD3 (John *et al.*, 1993). They frequently control plant development by regulating each other's abundance. It is thought that auxin and auxin conjugates may play a role in regulating the metabolism of cytokinin conjugates. However, little is known about how auxin and cytokinin interact at the signal transduction level (Swarup *et al.*, 2002).

Aims (1.8)

Auxin has long been suspected of involvement in plant responses to virus infection (see Pennazio and Roggero, 1996). However previous investigations have not produced any definitive evidence as to its role. This has been partly due to the limited nature of the experimental techniques available at the time when the majority of the early work was undertaken. The main problem with these approaches was that although such bioassays are able to show gross differences in auxin levels when comparing infected and uninfected plants, they do not provide any link to the causes of these differences. Also many of these techniques are only semi-quantitative, it can be difficult to distinguish IAA from inactive auxin analogues. In order to investigate the role that auxin plays in response to viral infection it is necessary to use better quantitative techniques to accurately measure the hormone levels, and more importantly to take advantage of the availability of mutants with lesions in auxin transport or responses.

The aims of this thesis are to establish whether auxin has a role to play during compatible plant-virus infection, to examine what this role is, and to determine how this fits in with the existing knowledge of plant virus-interactions.

Chapter Two

Chapter 2: Materials and Methods

Seed Stocks and Virus Isolates (2.1)

Arabidopsis *thaliana* variants were obtained from a local collection and contained the genotypes detailed below in **Table2.0.**

Seed Stock	Supplier	References
Col-0	Nottingham Arabidopsis Stock Centre (NASC)	www.arabidopsis.info
Ler *gl1*	NASC	www.arabidopsis.info
Ws	NASC	www.arabidopsis.info
Aux1-100	Alan Marchant	Bennett *et al.*, 1996
Aux1-7	Alan Marchant	Pickett *et al.*, 1990
Aux1-22	Malcolm Bennett	Marchant and Bennett, 1998
Tir1-1	Malcolm Bennett	Ruegger *et al.*, 1998
Tir2-2	Malcolm Bennett	www.arabidopsis.info
Tir3-1	Malcolm Bennett	Ruegger *et al.*, 1997
A7	Joel Milner	Love *et al.*, 1998
B6	Joel Milner	Love *et al.*, 1998
D4-2	J. Schoelz	Schoelz *et al.*, 2003
LAX2::GUS	Malcolm Bennett	Parry *et al.*, 2001

Table2.1 List of genotypes used in studies with details of the original supplier and relevant references.

Plant growth conditions (2.2)

Soil preparation (2.2.1)

Peat based potting compost (Levington Horticulture Ltd.) was autoclaved for 1 hour at 121°C in order to eliminate insect and fungal pests. After cooling, a solution of Intercept systemic insecticide (Levington Horticulture Ltd.) at $0.2gL^{-1}$ was used to moisten the soil prior to its use.

Arabidopsis (2.2.2)

Arabidopsis seeds were scattered onto soil filled trays, covered with cling film and left in darkness at 4°C for 2-4 days. Stratification promoted synchronised growth and development. Trays were then maintained at 21 ±1°C under 10 hours daylength. Osram "warm white" fluorescent tubes provided light at an intensity of 120μ $E/m^2/s$. These conditions have been shown to be optimal for the development of symptoms after virus inoculation (Cecchini *et al.*, 1998).

Virus isolates (2.3)

Virus stocks (2.3.1)

CaMV isolate Cabb B-JI (Delseny *et al.*, 1983) was originally provided by Dr. Simon Covey at the John Innes Centre (JIC) in Norwich. This isolate is described in Al-Kaff and Covey (1994, 1995) and in the VIDE database (http://biology.anu.edu.au/Groups/MES/vide/) . CaMV isolate CM4-184 can also be found on the VIDE database and is described by (Howarth *et al.*, 1981).

Production of virus stocks (2.3.2)

The virus stocks were maintained as desiccated leaf tissue from infected plants. Working virus solution was derived from the stocks via propagation in turnip and purification according to Hull *et al.*, (1976) and Gardner and Shepherd (1980). Approximately $1cm^2$ of desiccated tissue was ground up in 300µl dH_2O, centrifuged and the supernatant removed. Celite was added to the supernatant and 10µl was rubbed on to the first true leaf of each turnip plant at the two true leaf stage of development. After 3-4 weeks, at which time symptoms had developed (vein clearing, chlorosis mosaics and stunting), leaf material was taken from strongly symptomatic turnip plants and was frozen at –80°C.

The infected turnip tissue to be used was washed and wiped and ground up in 2-4-ml/g ice cold grinding buffer (0.5M KH_2PO_4, pH 7.2 and 0.75% (w/v) sodium sulphite) in a chilled blender. Urea was added to 1M along with 10 %(v/v) Triton X-100 to a final concentration of 2.5%. The mixture was then covered and left to extract overnight while gently stirring at 4°C.

The next day the ground up tissue was centrifuged at 5,000x g for 10 minutes at 4°C. The supernatant was then strained through 4 layers of muslin and spun at 25,000x g for 2 hours at 4°C in a Sorvall T865 rotor. The virus pellet was re-suspended in dH_2O and shaken at 4°C for a further 1-2 hours. The suspension

was then centrifuged at 7,000x g for 10 minutes at 4°C. The supernatant was decanted and spun down at 45,000 rpm in a Sorvall T865 rotor for 1 hour at 4°C.

The virus pellet was then re-suspended in TE buffer and stored in aliquots at –80°C. The concentration of virus in the aliquots was estimated spectrophotometrically at 260nm and 320nm (Gardner and Shepherd, 1980). The viability of each virus preparation was tested by inoculating turnip (Section 2.3.3) with 1μg of virus per plant and observing symptoms.

Virus infection (2.3.3)

Prior to inoculation, Arabidopsis seedlings were thinned to a density of 2 to 3 plants per cell. Plants at the 2-true leaf stage were mechanically inoculated by rubbing 2μl (100ng) of purified virus on one side of the first emergent true leaves (leaf 1 or 2) using celite as an abrasive. Mock-inoculated plants were inoculated with autoclaved water and celite.

Assessment of Symptom Response (2.4)

Digital photographs were taken using a Nikon Finepix S1 Pro digital camera fitted with a 28-135 mm lens. For assessment of symptoms photographs were taken of trays of plants and at higher magnification of individual plants for one control and two infected plants from each mutant line (Cecchini *et al.*, 2002).

Quantification of Virus Accumulation (2.5)

DNA Extraction (2.5.1)

DNA extraction was carried out using the Nucleospin Plant Kit (BD Biosciences, UK) as per the manufacturer's instructions. As the DNA was to be used in virus titre studies it was necessary to include a Proteinase K step in the protocol to allow the extraction of DNA from virus capsids (Cecchini *et al.*,2002). This is an important step as CaMV DNA can be found within infected tissue in both encapsidated and unencapisdated forms. Sixteen µl of Proteinase K (20mg/ml stock solution) was added to the samples, which were then shaken, before being left to incubate at room temperature for 3 hours. An extra centrifugation step was also added before the elution of the DNA. Samples were centrifuged for an additional 3 minutes at 11,000xg in a fresh collection tube to ensure that all traces of ethanol from the previous step were removed.

DNA gel electrophoresis (2.5.2)

DNA samples were separated by gel electrophoresis on 1.0% agarose gel in 0.5x TBE buffer (Sambrook and Russell, (2001); Appendix 1.17) as described in Sambrook and Russell, (2001), section 5.10. Samples were mixed with a loading dye (#161-0767, BIO-RAD, UK), 1 part dye to 5 parts sample, then loaded into the wells on the set gel. A 100bp ladder (#170-8352, BIO-RAD, UK) was also loaded onto the gel as a marker. Ethidium bromide-stained gels were photographed under UV illumination using a BIO-RAD gel documentation system.

Quantification of dsDNA using the PicoGreen Quantification Kit (2.5.3)

DNA samples extracted from both wild-type and mutant Arabidopsis samples were quantified using the PicoGreen dsDNA Quantification Reagents and Kits (Molecular Probes Europe BV, The Netherlands) as stated in the manufacturers' instructions. 1µl of sample DNA was used in the protocol along with the low-range standard curve.

Real time QPCR with DNA using SybrGreen (2.5.4)

Real time quantitative PCR was carried out using the Stratagene MX4000 thermocycler and Stratagene Brilliant SYBR Green QPCR Master Mix (#600548 Stratagene, California, USA) as described by Love *et al* (2005) . Firstly the experimental reaction was prepared. A master mix was made containing the sense and anti-sense primers for the gene of interest, water and SYBR Green 1 dye. The volumes of each, per sample were as follows:

12.5µl 2x SYBR Green 1

0.5µl Sense primer (10pmol/µl)

0.5µl Anti-sense primer (10pmol/µl)

9.5µl PCR-grade water

The master mix was then dispensed into each of the appropriate wells followed by 2µl of the experimental DNA. As well as the samples to be assayed, standards were also prepared. For viral DNA quantification, a standard stock of DNA extracted from Col-0 plants with known virus concentration was used for all experiments (Love *et al* 2005). This was serially diluted to give aliquots

containing from 100pg to 0.032pg. A no template control was also included to check for contamination of the reagents. The reactions were gently mixed and centrifuged briefly to remove any bubbles that could interfere with the fluorescence detection. The reactions were placed into the MX4000 instrument and the appropriate PCR program run. Depending on the primer sets used the temperature and cycle number was adjusted to provide optimal conditions. For viral DNA the PCR conditions were 10 min at 95°, then 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. The CaMV BJI 1 to 7 primers ($^{5'}$TACGCCAACTTCGACTCTCA $^{3'}$; $^{5'}$TGCTCGCTTTGGGTATTTTC $^{3'}$) amplify a 141 bp sequence across the ORF VII/ ORF I boundary and the rDNA control, the primers ($^{5'}$CGTGATCGATGAATGCTACC$^{3'}$; $^{5'}$GGGGTTTGTTGCACGTATTA$^{3'}$) amplify 199 bp of sequence in the Arabidopsis 18S rRNA gene (Love *et al.*, 2005). Because the SYBR Green 1 dye is light sensitive solutions containing the dye were stored in the dark until use.

To correct for any possible errors in estimating DNA concentrations in the samples, part of the gene encoding the Arabidopsis 18S rRNA was used as an internal reference standard (Love *et al* 2005). For each sample, the concentrations of CaMV DNA and 18S rDNA were estimated using the software provided by Stratagene. Final CaMV DNA concentrations were then expressed in arbitrary units, normalized to the amount of 18S rDNA in the sample

Northern Analysis (2.6)

RNA Extraction for Gene Expression (2.6.1)

The extraction of RNA from plant tissue was carried out using TRI Reagent, (Sigma-Aldrich. Inc, #T9424, UK) as outlined in the manufacturers instructions. An extra wash with ethanol was added to ensure that the RNA was clean enough to be used in subsequent RT-QPCR reactions. All samples were stored at –80°C until required for further analysis.

Removal of DNA from RNA Samples (2.6.2)

Contaminating DNA was removed from RNA preparations using the DNA-*free* kit supplied by Ambion (Europe) Ltd as per the manufacturer's guidelines.

RNA Gel Electrophoresis (2.6.3)

Before the sample can be run on a gel the gel tank, gel tray and the combs to be used should be wiped with a dilute NaOH solution. RNA samples are run on a 1.3% formaldehyde gel. The agarose was heated with water until it dissolved fully and then allowed to cool to around 60°C, then in the fume hood formaldehyde and 10x MOPS were added and the solution swirled gently to mix. This was then poured into the gel tray and allowed to set, which can take at least 45 minutes.

The RNA samples were heated at 65°C for 10 minutes before being mixed with loading dye (1part dye to 5 parts RNA) and loaded onto the gel. The running buffer was 1xMOPS and the gels are run at 50V.

Quantitative Slot-Blot Hybridisation (2.6.4)

The slot-blots were set up using DNase treated RNA extracted using the TRI Reagent method (2.6.1). The first stage of this protocol is sample preparation. A loading buffer was prepared as follows:

660 µl formamide

210 µl 37% (w/v) formaldehyde

130 µl 10x MOPS buffer (Sambrook and Russell, 2001; Appendix 1.18)

A series of microcentrifuge tubes were set up and to these the RNA samples and the loading buffer was added in the ratio 1 part RNA to 3 parts loading buffer. The samples were heated at 65°C for approximately 10-15 minutes to denature the RNA. They were then removed from the heat and placed immediately on ice. Once this stage had been reached the samples could be left on ice for as long as necessary until required.

The second stage of this protocol was to assemble the slot-blot manifold that holds the nylon membrane in place and also allows the samples to be drawn through the membrane using a small vacuum. The manifold consisted of 2 pieces

of 3MM paper and 1 layer of membrane. The manifold should only be handled while wearing gloves to avoid contamination.

Once assembled the samples were loaded. Taking one sample at a time 200ul of 20x SSPE was added and gently mixed before being loaded straight into the well. This was repeated until all of the samples had been loaded.

The manifold was then allowed to sit for 30 minutes or so to allow the samples to go through the membrane. After this time a pipette pump was used to apply a small vacuum to the manifold to draw any remaining samples onto the membrane. Then the manifold was dismantled, the membrane was wrapped in 3MM paper and left to air dry. When dry a cross-linker was used to fix the RNA onto the membrane, which was subsequently stored at 4°C until required.

Hybridization and Detection (2.7)

Prehybridization/Hybridization of Membrane (2.7.1)

The membranes were prehybridized in Northern Max Prehybridization/Hybridisation buffer (#8677, Ambion, Inc, USA). This was accomplished by placing the membranes in the NorthernMax buffer (20ml of buffer per hybridization tube) for a minimum of 2 hours at 42°C. Following prehybridization, the radiolabelled probe was denatured by boiling at 100°C and then combined with the buffer. The membranes were then left to hybridize overnight at 42°C.

Quantification of autoradiographs (2.7.2)

The total amount of nucleic acid loaded onto each lane was quantified by pixel analysis of digitally reversed images of fluorescence (Cecchini *et al* 2002), using QuantiScan (Biosoft, Cambridge, UK). Autoradiographs were scanned on a flatbed scanner, digitised and hybridisation was quantified by pixel analysis using QuantiScan (Cecchini *et al* 2002). To correct for any lane-to-lane variations in the quantity of RNA loaded, for each lane, the value of hybridising RNA was divided by the value for total RNA. RNA expression levels (in arbitrary units) are thus presented relative to the amounts of total RNA. Statistical analysis was carried out by ANOVA using Minitab version 13.20 (Minitab Inc., Pennsylvania, USA).

DNA Probes (2.8)

PCR (2.8.1)

DNA fragments required as probes were amplified from wild-type DNA using the HotStar Taq PCR Master Mix Kit (Qiagen, Crawley, UK) as detailed in the accompanying handbook. Primers were designed using the Primer 3 website (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences are shown in **Table2.2**.

Probe	Sense	Antisense
GH3	GCACTTATGAACCCGTTGT	GTGGAAGCACCTTGTGAG
SAUR_e	GCTTAAACAATGGCAGAAAA	AGGAAGTAACGGACGAGATT
SAG21	AGATTTCTTGGGTTCCAGAT	AAACCAAACGCTTCTCTATTT

Table 2.2 Sense and Antisense primer sequences as used in RT-QPCR and Northern Hybridisation.

Cloning (2.8.2)

PCR products to be used as probes in gene expression studies were cloned into E.coli cells using the TOPO TA Cloning Kit and OneShot TOP10 competent cells (# K4500-01, Invitrogen) The procedure was performed according to the protocols supplied with One Shot TOP10 cells.

Bacterial culture and plasmid isolation (2.8.3)

The *E. coli* glycerol stocks containing the vectors were grown in LB broth containing 50 µg/ml ampicillin according to Sambrook and Russell (2001); A2.2, A2.6. Bacteria were pelleted by centrifugation at 4000 rpm for 5 mins in a microcentrifuge, and re-suspended in STE (Sambrook and Russell, (2001) A1.22). Plasmids were extracted using the QIAprep Spin Miniprep Kit Protocol (Qiagen, West Sussex, UK). The last step of the protocol was modified, whereby the EB buffer was diluted 1 in 4 and heated to 70°C before addition to the column.

Liquid and Solid Bacterial Media (2.8.4)

Luria-Bertani Medium (LB) was prepared as outlined in Sambrook and Russell, (2001) Appendix 2.2, and for solid media agar was added, 15g/L as detailed in Sambrook and Russell, (2001) Appendix 2.5.

Purification of PCR fragments (2.8.5)

PCR fragments were separated by gel electrophoresis according to the protocols described by Sambrook and Russell, (2001) Section 5.14-5.17. The ethidium bromide-stained bands were visualized using a 302 nm UV illuminator, and the desired fragments were identified by comparing against 100bp and 1kbp ladders (BIO-RAD, UK). The appropriate bands were excised and the fragments were purified using a QIAprep Spin Miniprep Kit (# 27104, Qiagen, Crawley, UK) as instructed in the manufacturers' handbook.

Radiolabelling of Probes (2.8.6)

Purified DNA fragment were labelled with 50 μCi ^{32}P-dCTP (3000Ci/mMol; Amersham, UK) using the Rediprime II Random Prime Labelling System (#RPN 1634, Amersham Biosciences, Buckinghamshire, UK) as described in the manufacturers' guidelines. The fragments were purified using a NICK Column (Pharmacia Biotech). The purified radiolabelled probe was boiled at 100°C for 10 minutes before adding to the filters that had already been pre-hybridised (2.7.1).

RT-QPCR (2.9)

cDNA Synthesis (2.9.1)

RT-QPCR was carried out according to Love *et al* (2005). The reverse transcriptase step was performed using AMV Reverse Transcriptase (#M5101 Promega Corporation, USA). Each sample contained:

1ng DNase treated RNA

1.25µl Oligo(dT)15 Primer (#C1101, Promega Corporation, USA)

PCR-grade water to give total of 15.9 µl.

This was then heated for 10 minutes at 70°C then immediately placed on ice. To each tube 9.1 µl of master mix (see Pg 64) was added and the tubes were placed back into the PCR machine (PTC) for 30 cycles under the following PCR conditions:

Master Mix: 1 µl AMV (10U/ µl)
0.625 µl RNasin (40U/ µl)
1 µl dNTPs (25mM)
5 µl AMV Buffer (5x)
1.5 µl PCR-grade water

Program: 48 °C for 45 minutes
95 °C for 5 minutes
4 °C forever

Reverse transcriptase Real-Time Quantitative PCR (2.9.2)

After cDNA synthesis the contents of each reaction was used as template in real time quantitative PCR (2.5.4). This was carried out as described previously (2.5.4) except that the standards used were plasmid DNA. Plasmids contained cDNAs for the following auxin-responsive gene, SAUR_e (see Section 2.8.2). Primers used were as follows:

SAUR_e $^{5'}$CGATTGTGACTCGGTGTGAG$^{3'}$

$^{5'}$CCAGGAAGTAACGGACGAGA$^{3'}$

ACT2 $^{5'}$CTAAGCTCTCAAGATCAAAGGCTTA$^{3'}$

$^{5'}$ACTAAAACGCAAAACGAAAGCGGTT$^{3'}$

Plant Growth Assays (2.10)

Seed sterilization (2.10.1)

A 1% Tween 20 solution was prepared and one PRESEPT tablet (Coventry Chemicals Ltd., UK) was added to 35ml distilled water in a beaker and allowed to dissolve. At this point 4-5 drops of 1% Tween 20 were also added. Once fully dissolved, 5ml of this solution was added to 45ml 95% ethanol. This was mixed and 1ml added to tubes containing 10-15mg of the seeds to be sterilized. The seeds were left to soak for approximately 5-10 minutes and then shaken gently and left for a further 6-7 minutes.

Seed sterilization was carried out in a laminar flow hood.

The solution was poured off each of the tubes of seeds, and replaced with 1ml of 95% ethanol. The tubes were shaken gently and the ethanol poured off. This was repeated a total of three times and once completed, the tubes were covered with nescofilm, leaving an air gap, and left to dry in the hood overnight (2-3 hours minimum).

Plant Growth Media (Solid) (2.10.2)

1/2 Murashigi and Skoog (MS) with 0.8% agar was prepared.

IAA Root Growth Assays (2.10.3)

Sterilized seeds for the following genotypes, Col-0, WS, aux1-100, aux1-7, MDR1, A7, B6 and ARC 1A, 1C, 2C and 2E, were sprinkled onto 1/2 MS plates and the plates sealed. The plants were left for 4 days in the cold room and then transferred to the growth room where they were incubated vertically for 5 days.

Half MS plates containing various concentrations (0nm, 1nM, 5nM, 10nM, 50nM and 100nM in 100% Analysis Grade Ethanol) of Indole-3-acetic acid (IAA) were prepared (Pickett *et al.*, 1990). Seedlings from the initial MS plates were transferred to the IAA containing plates and arranged in a line. Plants were selected of roughly the same size, and a line was marked on the plate where the roots ended. The plates were sealed and incubated vertically in the growth room for 5 days. The roots were then measured from the previous line onwards and any root branching taken into account. The measurements were done using.

TIBA Growth Assay (2.10.4)

The responses of various Arabidopsis auxin mutant and CaMV gene VI transgenic lines to the auxin efflux inhibitor 2, 3, 5-triiodobenzoic acid (TIBA) were assayed. Sterilized seed (2.10.1) for each of the various lines were sprinkled onto the surface of ½ MS plates containing TIBA at concentrations ranging from 0μM to 35μM. The plates were sealed and stored in the dark at 4°C for 1-2 days before being transferred to a short day growth room with a photoperiod of 10 h or less, and grown at 20°C. The plants were visually assessed at 16 days and 24 days after being sown (2.4); also at each of these time points duplicate tissue samples were harvested (the contents of one plate). The samples were frozen in liquid N_2, ground to a fine powder and RNA extracted (2.6.1). Each sample was then analysed using RT-QPCR.

Analysis of Virus Movement (2.11)

Virus movement was followed using a recombinant CaMV engineered to express GFP as a free protein (Turner and Covey, unpublished) . CaMV-GFP, which expresses GFP as a free protein in the cytosol, was constructed by substituting ORFII of Cabb B-JI with a modified M-GFP5 (Chalfie *et al.*, 1994). The full length CaMV-GFP DNA sequence was inserted into pBluescript using the unique *SalI* site at nucleotide 4831 of the Cabb B-JI sequence, and was maintained and propagated as plasmid DNA. For infections, plasmid DNA containing the CaMV-GFP sequence was digested with *Sal*I to release the insert.

Biolistic Particle Delivery System (2.11.1)

Because CaMV-GFP shows poor infectivity when manually inoculated onto Arabidopsis plants were infected by particle bombardment using a protocol developed by V. Laval (Laval *et al*, submitted). This was carried out using the PDS-1000/He particle gun (BIO-RAD, UK) as per the manufacturers' instructions. The bombardment chamber itself and all of the holders, rupture disks, stop screens and carriers were cleaned with 90% ethanol and all procedures were carried out in a laminar flow hood.

Twenty-five µl of sterile water was added to a microcentrifuge tube and placed on ice along with a supply of 100 % ethanol. The components required for sample preparations were removed from the freezer and allowed to thaw completely ready for use. A tube containing 40mg BIO-RAD submicron gold particles (0.6µm) was sonicated for 1 minute in a sonicator bath (Grant Instruments Ltd, Cambridge, UK). Then 25µl of gold was added to the tube of sterile water and sonicated again for 1 minute. The sample was taken back to the hood and either 5µl sterile water was added (control samples) or 4 µl sterile water and 1µl CaMV: GFP DNA (118 µg/ml) was added. Virus samples contained 0.118 µg/µl. It is important at this stage to keep the virus and water away from the gold until they have been mixed. To this mixture 50µl 2.5M CaCl2, 20µl 0.1M Spermidine was added to the lid of the tube and mixed together. Once mixed the tube was capped and lightly vortexed. The tube was left on the bench for 3 minutes, lying on its side then centrifuged for 3 seconds at 13,000 rpm. The supernatant was removed and the pellet re-suspended in 150µl

of chilled 100% ethanol. The suspension was centrifuged again for 3 seconds at 13,000 rpm. The supernatant was removed and re-suspended in 85µl of chilled 100% ethanol. The samples were then placed on ice until required.

The holders were placed in a petri dish and using forceps, the carrier disks were placed into the holders. A red plastic cup, which is provided with the chamber, was used to fit the disks neatly into the holders. The gold/virus DNA or gold only samples were vortexed briefly, 8µl was placed into the centre of the carrier disks, and allowed to dry. A new rupture disk was placed into the holder and screwed into the chamber and a stop screen was positioned into the bottom of the bombardment platform. Once dried, the carrier disk was inserted into its holder and the whole platform slotted into the chamber. Cells (40 x 40 mm) each containing two plants at the eight leaf stage were then placed in the centre of the chamber underneath the platform and a vacuum of approximately 28 psi is applied. When ready, the fire button was pressed until the disk was ruptured.

Following bombardment, the plants were sprayed with water to help recovery and placed to one side.

Confocal Laser Scanning Microscopy (2.11.2)

Visualisation of GFP from CaMV:: GFP plants was achieved using the LSM 510 laser scanning microscope (Carl Zeiss Ldt., UK). Prior to using the microscope the tissue samples were prepared and mounted onto slides. Two lines of adhesive were placed down the width of the glass slide and allowed to air dry. The tissue to be examined was harvested and de-gassed using a syringe. The tissue was then mounted onto the slide, covered with a cover slip and a small amount of water added under the cover slip. The slide was then viewed under the microscope with the Argon/2 laser at the following settings:

NFT 545nm , BP 560-615

HFT 488 nm

Excitation 10 % at 488 nm

Laser Intensity 75%

Scan Speed 1.60µS

Frame size 1024 x 1024

	Channel 2	**Channel D**
Pinhole	2.95	n/a
Gain	756	190
Amp Offset	-0.029	-0.03
Amp Gain	1	1
BP	505-530	

Western Blot Analysis of Proteins (2.12)

Analysis of proteins was carried out essentially as described by Sambrook and Russell, (2001), Appendix 9.28.

Sample preparation (2.12.1)

Plant tissue samples were ground to a fine powder in liquid nitrogen. Then 200 µl solubilization buffer (detailed below) was added and the samples were ground on ice and then vortexed. The solubilized tissue centrifuged for 20 minutes at 13,000 rpm and 2 °C and the supernatant collected.

Solubilization buffer: 1.25ml 1M Tris pH 7.5

1.5ml 5M NaCl

0.25ml 1M $MgCl_2$

5ml Glycerol

42ml H_2O

Assay of Protein Concentration (2.12.2)

Protein concentration was assayed according to the procedure of Bradford (1976) according to the manufacturers protocol, using a BIO-RAD DC Protein Assay, kit (BIORAD, USA).

SDS-electrophoresis Gel (2.12.3)

Proteins were separated prior to Western Blotting by electrophoresis on SDS gels as described by Sambrook and Russell, (2001), Appendix 8.42. The 10% SDS-PAGE resolving gel contained 1.875ml acrylamide solution (40%; 37.5:1 acrylamide: N'N' methylene bisacrylamide) the 5.5% stacking gel contained 250ul acrylamide solution (40%; 37.5:1 acrylamide: N'N' methylene bisacrylamide). The resolving gel was poured first and then the stacking gel and left to set. To run the gel 10µl of protein (see section 2.12.1) was added to 10µl of 2x SDS loading buffer (detailed below). The protein was then denatured by heating at 100 °C for 5 minutes, and then centrifuged at 13,000 for 5 minutes. The samples were then loaded onto the gel and electrophoresed at 200 V for 50 – 60 minutes.

2x SDS loading buffer: 125mM Tris-HCL pH6.8

4% SDS

20% Glycerol

10% β-mercaptoethanol

0.008% bromophenol blue

Protein Transfer (2.12.4)

The stacking gel was cut off and the stacking gel incubated with 1x Transfer Buffer (200ml 10 x Transfer Buffer (61g Tris, 278g Glycine, 2L dH_2O, pH 8.3)

400ml Methanol and 1400 dH_2O) for 5 to 10 minutes. The nitrocellulose membrane, sponge, and Whatman paper were soaked in 1x Transfer Buffer to remove air bubbles and then the transfer sandwich was assembled in a BIO-RAD mini-protean® 3 Cell (BIO-RAD, USA). The transfer sandwich was then submerged in the transfer tank filled with transfer buffer in the cold room and left for 50 minutes at 100 volts under agitation.

Immunological Detection of Proteins (2.12.5)

Immune detection was carried out using a rabbit anti-P6 polyclonal antibody (a kind gift from Dr J Schoelz, Missouri, USA). The antibody was raised against P6 from CaMV isolate W260 (Yu *et al.*, 2003). The membrane was blocked prior to the addition of the primary antibody in a blocking buffer (8g dry skimmed milk in TBT (50mM Tris and 1% Tween 20, pH 8.0) + 0.1% Triton X-100) for 1 hour. The milk was then removed and fresh buffer added along with a 1/2000 dilution of antiserum. This was left to incubate at room temperature for 1-2 hours. The primary antibody was then removed and the membrane washed for 5 minutes in 1x TBS. TT ((150mM NaCl and 10mM Tris HCl pH 7.5) + 0.2% Triton and 0.05% Tween) twice and then washed once in 1x TBS (150mM NaCl and 10mM Tris HCl pH 7.5).

A 1/5000 dilution of the secondary, anti-rabbit antibody was added to fresh blocking buffer and allowed to incubate for 1 hour. After this time the buffer/

antibody solution was removed and the membrane washed four times in 1x TBS.TT for 5 minutes each time followed by a single wash in 1x TBS. The membrane was then exposed briefly with autoradiography film in the dark room and then developed.

The protein-antibody complexes were detected using ECL Plus western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) as described in the manufacturers' instructions.

Genetic Crossing of Arabidopsis (2.13)

Crosses were carried out using a standard procedure adapted from Weigel and Glazebrook, 2002 (Arabidopsis: A Laboratory manual). In order to carry out cross fertilization between Arabidopsis mutant and transgenic lines, plants were grown under long day conditions in a greenhouse until the inflorescences had developed and the flowers were at a stage where they were not open but not tightly closed, just before the white petals became visible. Any flowers that were too old or young were removed from the inflorescence leaving approximately 2-6 flowers for pollination. Each flower was carefully stripped back using forceps to leave the intact ovary. Pollen was obtained from a donor plant and gently rubbed onto the top of the exposed ovary. This was repeated at least twice to ensure proper pollination. The ovary was then covered and labelled to show which cross had been performed. Once all of the desired crosses had been carried out the plants were returned to the greenhouse and allowed to develop until the siliques had reached maturity at which point the seeds were harvested.

Chapter Three

The effect of auxin on CaMV infection within *Arabidopsis thaliana*

Introduction (3.1)

The type and severity of the symptoms associated with disease are dependent on both the virus isolate and the host plant involved. The severity of symptoms is not simply dependent on the virus titre within the infected plant, but on a number of factors. Environmental conditions, such as light intensity and temperature (Cecchini *et al.*,1998; Cecchini *et al.*, 2002; Lee *et al.*, 1996), can influence the symptom response of a host plant, as can changes in the levels and distribution of plant hormones within the plants (Qiu *et al.*, 1997; Cecchini *et al.*, 2002). Interactions between components of the virus and host genomes lead to the induction of a set of pre-programmed responses within the host (Covey *et al.*, 2000; Johal *et al.*, 1995). Symptoms can differ widely between hosts but common features of systemic virus infections include chlorosis, leaf malformations, mosaic patterns, stunting and a reduction in yield. The first response to be observed is often the development of local lesions at the site of initial infection. These lesions may appear as chlorotic patches on the inoculated tissue. The virus then moves into the surrounding tissue and spreads through the vasculature to establish a systemic infection. It is the symptoms of a systemic infection, such as stunting and chlorosis that are the root cause of economic losses (Hull R. 2001).

It has been suggested that the physical symptoms that develop during infection are a result of virus-induced alterations in physiological, biochemical and metabolic processes within the plant (Reviewed by Pennazio and Roggero, 1996). Changes in the levels and distribution of plant hormones within infected plants are likely to be, at least in part, responsible for these responses. It is well known that the hormones ethylene, JA and SA have important roles in plant defence, against a number of different pathogens (Grant and Lamb, 2006). However, much less is known about the roles, if any, that the 'classical' plant hormones, such as ABA, GA, cytokinin and auxin have in this process. Historically, studies attempting to establish the functions of these hormones have been linked by the lack of accurate, sensitive techniques to assay the levels and distribution of these hormones and have therefore resulted in misleading and inconsistent results (Jameson, 2002).

In recent years, studies carried out to establish the roles of these hormones in response to viruses and other pathogens have been limited, and clear roles during infection have yet to be identified. In the past there has been suggestive evidence to indicate that auxin might be involved in the symptom response (Reviewed by Pennazio and Roggero, 1996). The symptoms observed during infection are often reminiscent of those observed in Arabidopsis mutants with defects in auxin signalling and transport. For example, auxin plays a key role in regulation of cell elongation and division (Bennett et al., 1998), and mutants with defects affecting these processes often show reduced growth and stunting reminiscent of the symptoms in virus infected plants.

Auxin signalling and transport is an area of research that has received considerable attention in recent years, and our understanding of the subject has been advanced greatly through the identification of Arabidopsis mutants that have defective auxin responses (Leyser, 1997). These mutants are now a valuable tool for studying the effects of a variety of different biotic and abiotic stresses on auxin signalling and transport.

Preliminary studies (Cecchini and Milner, personal communication) had pointed to a possible role for auxin in the response to CaMV infection. Infection studies using auxin mutants, *aux1* (Pickett *et al.*, 1990), *axr2* (Timpte *et al.*, 1994) and *axr4* (Hobbie and Estelle, 1995) suggested that *aux1* alleles showed changes in the symptom response to some isolates of CaMV. *axr2* and *axr4* were indistinguishable from the wild-type. This pointed to a possible involvement of auxin signalling during virus infection. The first objective of this work was to identify auxin mutants that showed alterations in response to CaMV infection compared to wild-type and to establish the effect of the mutation on virus replication and/or spread within the host plant. For example, how is virus accumulation affected? Do defects in auxin signalling and transport affect the movement and spread of the virus and ultimately its ability to establish systemic infection within a host?

Results (3.2)

Symptom Phenotypes and Virus Accumulation After Infection With CaMV in Arabidopsis mutants (3.2.1)

Auxin signalling and transport mutants and the corresponding wild-type ecotypes were manually inoculated with two isolates of CaMV, CM4-184, which causes moderate symptoms in Arabidopsis and Cabb B-JI, which causes very severe symptoms (Cecchini *et al.*, 1998). Infections were carried out with two different virus isolates because preliminary studies (Cecchini and Milner, unpublished) had suggested that, depending on the mutant phenotype, differences in symptom response might be easier to identify with one or other of a severe or mild isolate (Cecchini *et al.*, 2002; Geri *et al.*, 2004). The symptom response of each of the mutant lines and wild-type plants were then followed on a day to day basis by visual inspection, and plants were photographed at regular intervals from the development of symptoms, typically 12 dpi, through until 24 dpi (days post infection).

AUX1 Mutant Alleles (3.2.1.2)

The first mutants to be studied were alleles of the *AUX1* gene since preliminary studies had suggested differences between *aux1* mutants and wild-type in response to CaMV infection (Cecchini and Milner, unpublished). The *Arabidopsis AUX1* gene has been identified as a member of the auxin amino acid permease (AAAP) family of proton driven transporters. Plant amino acid permeases facilitate the uptake of amino acids into plant cells; Bennett *et al.*

(1996) suggested that *AUX1* performed a similar transport role, facilitating the movement of the amino acid-like signalling molecule, IAA.

Approximately fifty mutant alleles of *AUX1* have been identified. It is important to use more than one allele when using a genetic approach since allele-specific differences can be eliminated. Three alleles were chosen for this work, *aux1-100*, *aux1-7* and *aux1-22* (See **Table 3.1**) along with their corresponding wild-type lines, Columbia (Col-0) and Wassilewskija (WS). One of these alleles, *aux1-100* produces no AUX1 protein, the other two are weak alleles in which a modified protein is produced.

Allele	Type of Mutation	Description
aux1-100	Deletion (T-DNA insertion)	No protein produced. Resistant to ethylene and auxin; agravitropic root phenotype and no obvious aerial phenotype. (Bennett *et al.*, 1996)
aux1-7	Point (G → A)	Single substitution, leading to Gly → Asp at position 459. Produces full length protein but with single amino acid substitution. Aerial portions of plant similar in appearance to wild-type; slight increase in root elongation and altered geotropic response; resistant to ethylene and auxin. (Marchant and Bennett, 1998)
aux1-22	Point (T → A)	Single nucleotide change at splice junction, leading to a deletion of 34 bp in the cDNA. Smaller protein than wild-type produced. Auxin and ethylene resistant roots; agravitropic roots. Leaves show slight curling. (Marchant and Bennett, 1998)

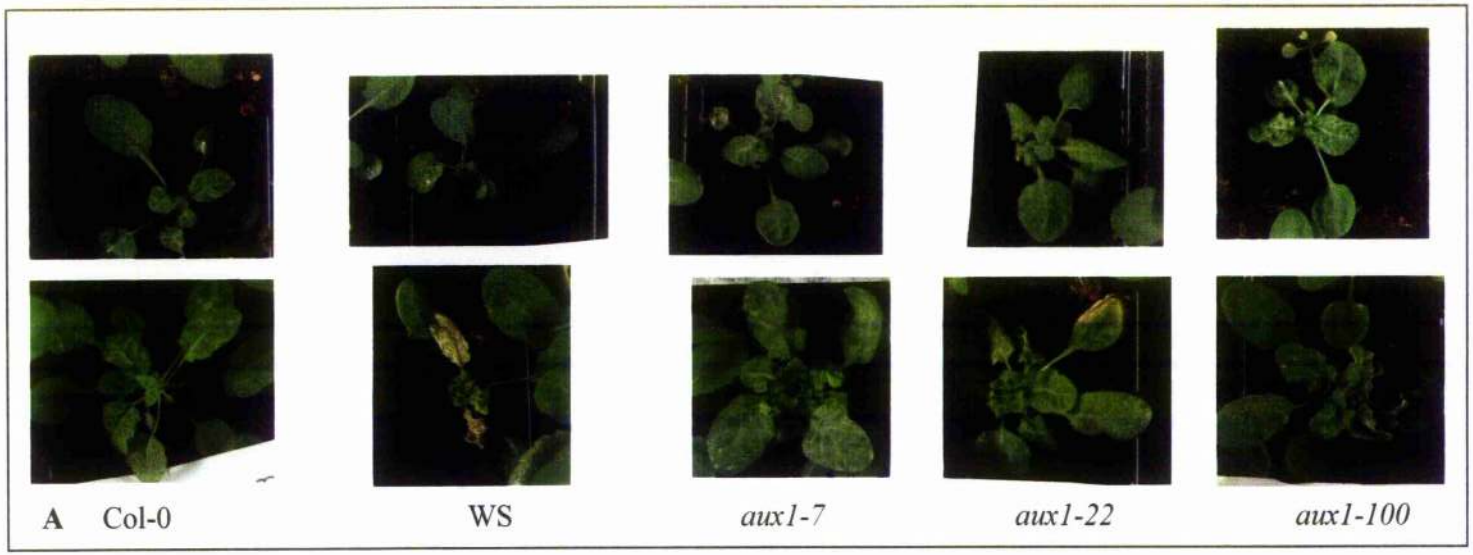
Table 3.1 Alleles of the AUX1 influx carrier.

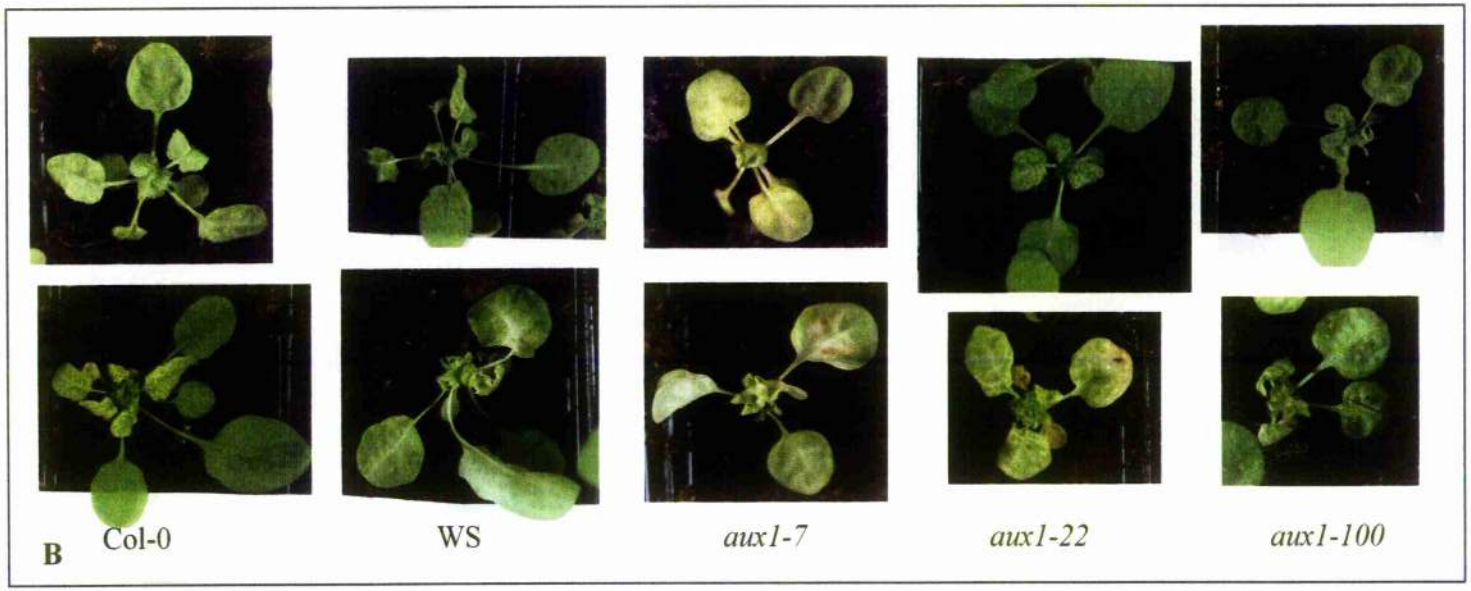
The typical symptoms (stunting, chlorosis, vein clearing, mosaics) associated with CaMV infection were observed in all genotypes (see **Figure 3.1**). As expected the less severe isolate CM4-184 induced slightly milder symptoms than with the severe isolate Cabb B-JI. These consisted primarily of stunting, leaf malformation, vein clearing, patches of chlorosis and the development of mosaic patterns. In CM4-184-inoculated wild-type plants, Col-0 and WS, local lesions were first identified at 11 dpi and systemic symptoms were first visible at 15 dpi for all three of the *aux1* mutant alleles and wild-types. Local lesions developed much earlier in the Cabb B-JI inoculated plants, typically from around 7 dpi in Col-0, WS and all three *aux1* alleles. Systemic symptoms were also observed earlier and appeared much more severe than those associated with CM4-184. The first mild systemic symptoms (e.g. vein clearing, chlorosis) were noted at 9 dpi and continued to increase in severity through until 28 dpi. At this point symptoms were so severe that the plants were very severely stunted, with nearly all of the leaf tissue being chlorotic. However, although slight differences were observed between the two isolates of the virus at early timepoints, in contrast to the preliminary results of Cecchini and Milner, there were no differences in the severity or quality of the symptoms that developed between the mutants and their corresponding wild-types.

Fig 3.1 Symptom response of *aux1* alleles and the corresponding wild-types, Col-0 and WS, to CaMV infection.

(A) Symptom response of the *aux1* mutant alleles and wild-types inoculated with CaMV isolate CM4-184.

(B) Symptom response of the *aux1* mutant alleles and wild-types inoculated with CaMV isolate Cabb B-JI.


15dpi
24dpi
A
Col-0
WS
aux1-7
aux1-22
aux1-100


15dpi
24dpi
B
Col-0
WS
aux1-7
aux1-22
aux1-100

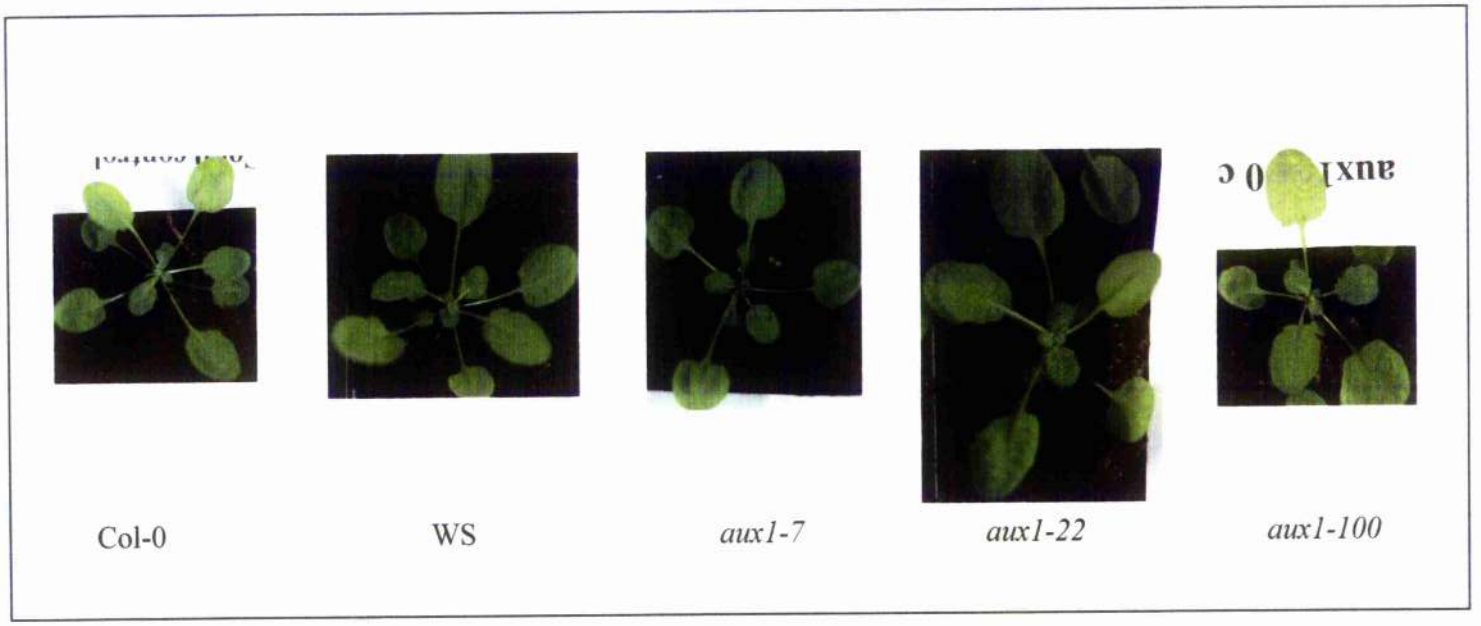


Fig 3.2 Uninfected control Arabidopsis wild-type and aux1mutant alleles.

Although no differences in symptom severity or timing were observed, symptom responses in CaMV infected Arabidopsis are not necessarily directly related to virus titres within infected plants (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002). Therefore to examine what effect if any, changes in auxin signalling and transport might have on virus levels, virus accumulation was compared in wild-type and mutants. Triplicate tissue samples were harvested from Cabb B-JI - inoculated and also mock inoculated control plants at 3 day intervals from 0 dpi until 28 dpi. Each sample was produced from a pool of three individual plants. Total DNA was extracted (see Materials and Methods 2.5.1) from infected plants and virus DNA was quantified using quantitative real-time PCR (See Materials and Methods 2.5.4). DNA was extracted using a Nucleospin Kit but the procedure was modified by the addition of Proteinase K. This results in release of virus DNA from assembled virus particles (Cecchini *et al.*, 2002). The virus DNA was quantified as a proportion of the total DNA extracted from the samples. Genomic DNA encoding part of the 18S ribosomal RNA was used as an internal standard and levels of virus DNA were normalized to levels of this ribosomal DNA. The QPCR data was analysed statistically using the General Linear Model (GLM) with Bonferroni analysis. Results are shown in **Figure 3.3.**

Virus levels were extremely low during the early stages of infection, from 0 dpi through to 8dpi, in all of the *aux1* alleles and their corresponding wild-types, Col-0 and WS. From 12 dpi onwards the virus levels began to rise rapidly until 21 dpi or 24 dpi ($p<0.05$). At 28 dpi a significant decrease ($p<0.05$) in virus was

observed in all of the lines except Col-0. *aux1-7* and *aux1-22* consistently showed slightly more virus accumulation than the wild-type Col-0. However, ANOVA indicates that these differences are not significant ($p>0.05$). *aux1-100* also closely followed the pattern of virus accumulation shown by its wild-type WS. Again ANOVA indicated that virus accumulation in *aux1-100* and WS are not significantly different ($p>0.05$).

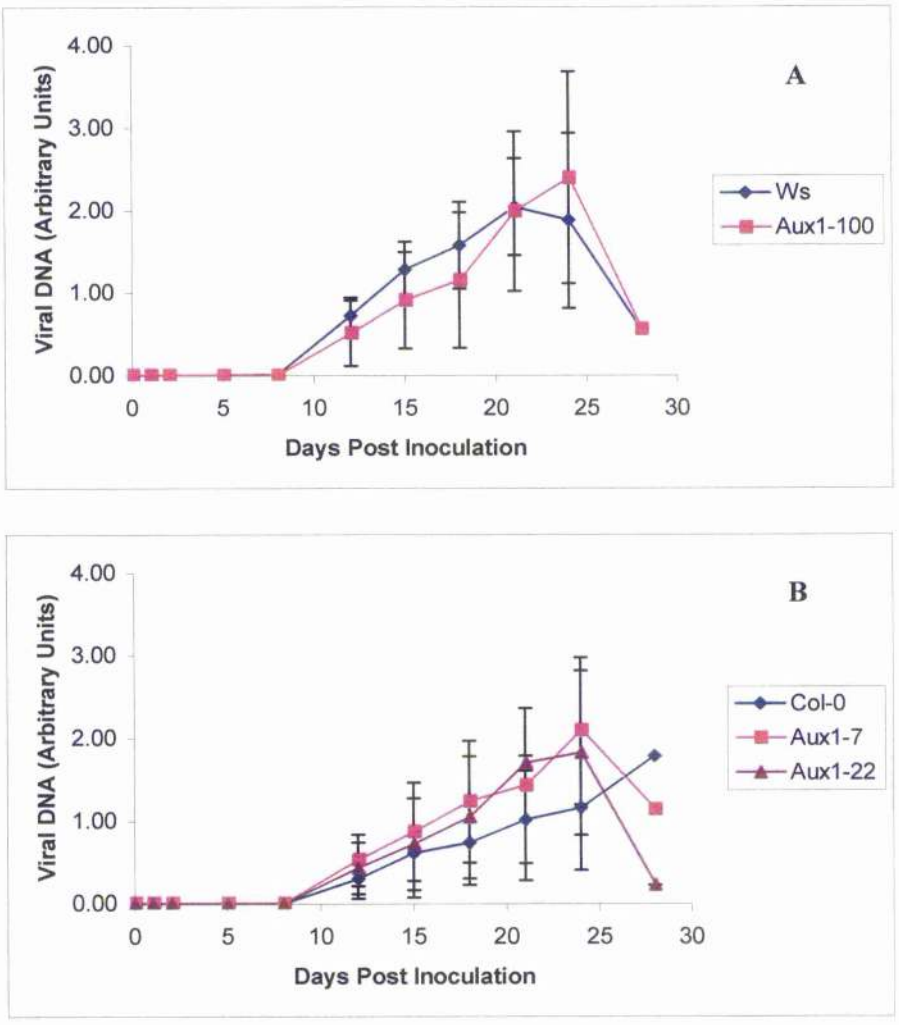


Fig 3.3 CaMV DNA Accumulation in auxin mutants and wild-type Arabidopsis. Levels of virus DNA in arbitrary units were normalized to levels of 18S ribosomal DNA. A value of 1.00 is equivalent to an average of 1400 molecules of CaMV per cell (Love *et al.*, 2005). Samples were taken at three day intervals from 0 dpi until 28 dpi. Error bars show standard errors (n=3).

(A) Levels of CaMV DNA in *aux1-100* and the corresponding wild-type WS.

(B) Levels of CaMV (Cabb B-JI) DNA in *aux1-7*, *aux1-22* and the corresponding wild-type Col-0.

Transport Inhibitor Response Mutants (3.2.1.3)

Three further auxin mutants were examined. These included the Transport Inhibitor Response mutants (TIR), which were identified from a screen for plants with altered responses to transport inhibitors (Ruegger *et al.*, 1997). The responses to CaMV infection of three mutants, *tir1-1*, *tir2-2* and *tir3-1* were examined (See **Table 3.2**). Plants were inoculated with CaMV CM4-184 and Cabb B-JI, the symptom responses were assessed and virus titres quantified as described previously for the *aux1* alleles.

Allele	Type of Mutation	Description	Type
tir1-1	Point (G→A at +440)	Semi dominant mutation;resistant to auxin inhibition of root elongation, deficient in auxin regulated growth processes, encodes a protein that contains an F-box domain and leucine rich repeats. Aerial phenotype is similar to wild-type.	Auxin signalling. Component of SCF-type ubiquitination complex. Auxin receptor. (Ruegger *et al.*, 1998; Kepinski and Leyser, 2005)
tir2-2	Unknown	Similar phenotype to *tir1*.	Auxin Signalling, not mapped.
tir3-1	Point (T→A at +7331)	Variety of morphological defects. Both auxin and NPA binding activity are dramatically reduced.	Essential for the synthesis, localization or function of the NPA binding site. Encodes BIG protein with homologies to Calossin/Pushover from Drosophila. Involved in protein trafficking (Gil *et al.*, 2001).

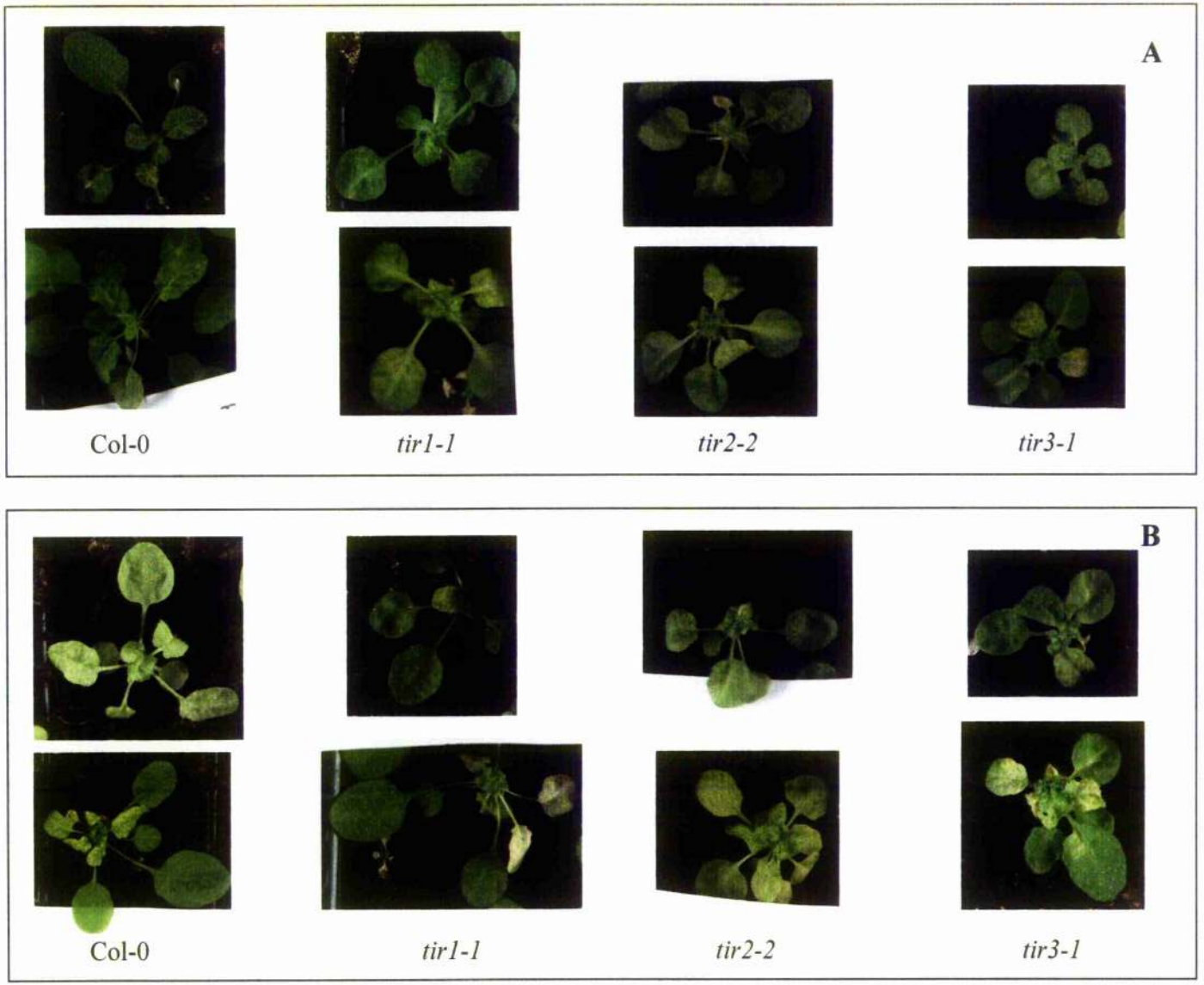
Table 3.2 Transport Inhibitor Response mutants used in infection studies.

Two of the three mutant lines, *tir1-1* and *tir2-2* developed the typical symptoms associated with CaMV- infection of their corresponding wild-type (Col-0; see **Figure 3.5**). In CM4-184 inoculated plants, local lesions appeared at approximately 12 dpi with systemic symptoms first observed at 14 or 15 dpi. However the *tir3-1* mutant, showed a consistent delay of 3 days in the appearance of both local and systemic symptoms compared to Col-0 (see **Figure 3.4, A**). When plants were inoculated with Cabb B-JI, symptoms appeared at a much earlier stage of infection than with CM4-184. In *tir1-1*, *tir2-2* and Col-0 local lesions were observed as early as 7 dpi, with systemic symptoms appearing after 10 dpi. The symptoms associated with Cabb B-JI infection were more severe than those associated with CM4-184, and by the end of the timecourse infected plants were very chlorotic and stunted. However, like with CM4-184, *tir3-1* plants inoculated with Cabb B-JI showed a delay of approximately 3 days in the onset of both local lesions and systemic symptoms compared to wild-type (See **Figure 3.4, B**). Symptoms were also much less severe in the *tir3-1* mutant. This consistent delay suggests that the *tir3-1* mutation may be affecting either the movement and/or replication of the virus.

Fig 3.4 Symptom response of *tir* mutants and the corresponding wild-type, Col-0, to CaMV infection.

(A) Symptom response of the *tir* mutants and wild-type inoculated with CaMV isolate CM4-184.

(B) Symptom response of the *tir* mutants and wild-type inoculated with CaMV isolate Cabb B-JI.


A
15dpi
24dpi
Col-0
tir1-1
tir2-2
tir3-1
B
15dpi
24dpi
Col-0
tir1-1
tir2-2
tir3-1

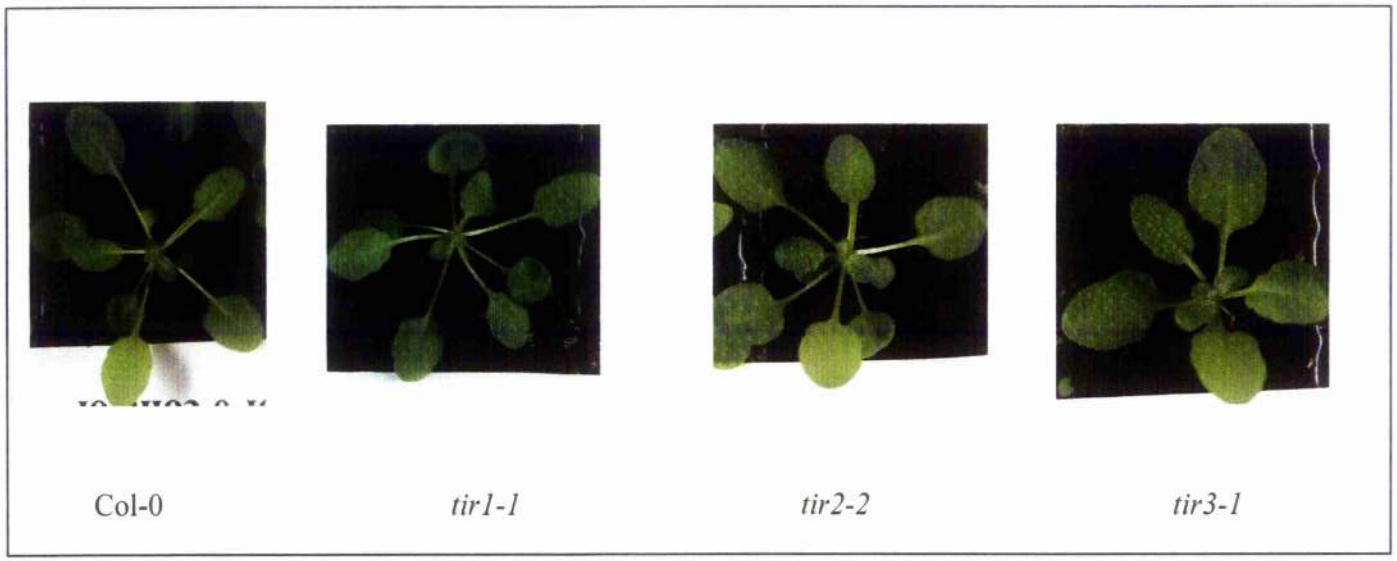


Fig 3.4 Uninfected control Arabidopsis wild-type and tir mutant plants.

To determine if virus levels were altered in any of the mutants, virus levels were compared with wild-type (see **Figure 3.6**). All plants contained almost undetectable levels at the early timepoints, 0 dpi through to 8 dpi, but from 12 dpi onwards the virus levels increased in all genotypes. As was observed with the *aux1* alleles, virus levels decreased after 24 dpi (ANOVA $p<0.05$). *tir1-1* virus levels appeared consistently higher than wild-type; ANOVA identified the p value to be 0.073. Although this is greater than the conventionally accepted figure of $p=0.05$, there is still a 93% chance that differences in virus levels in *tir1-1* compared to wild-type, are an authentic consequence of the mutation. There were also no significant differences in virus DNA levels between Col-0 and the *tir2-2* mutants ($p>0.05$). In contrast, *tir3-1* showed a consistent delay in virus accumulation and ANOVA identified *tir3-1* virus levels as being significantly lower than either Col-0 or *tir1-1* ($p<0.05$). Therefore in *tir3-1*, virus accumulation is delayed compared to wild-type and virus titres throughout the infection are lower. This delay in virus accumulation most likely corresponds with the delay seen in the development of symptoms in the *tir3-1* mutants. Thus mutations to a gene with a role in auxin efflux, impinge significantly on virus infection.

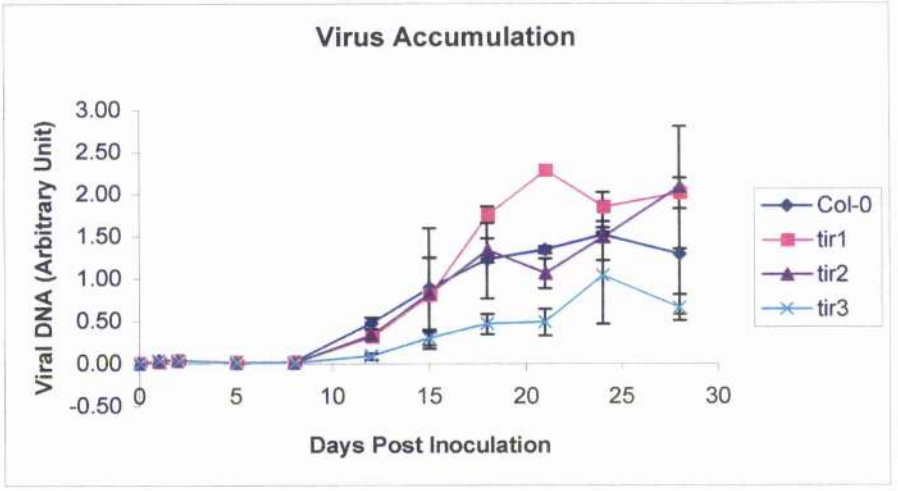


Fig 3.6 Levels of CaMV (Cabb B-JI) DNA present in mutant and wild-type. Levels of virus DNA in arbitrary units were normalized to levels of 18S ribosomal DNA. A value of 1.00 is equivalent to an average of 1400 molecules of CaMV per cell. Samples were taken at three day intervals from 0 dpi until 28 dpi. Error bars show standard errors (n=3). The symbols for each genotype are indicated in the figure legend.

Impact of Auxin Signalling on the Movement and Spread of CaMV (3.2.3)

tir3-1 shows a very significant reduction/delay in virus accumulation; this could be a result of reduced replication at a cellular level, reduced cell to cell movement, or a reduction in long distance movement. To distinguish between these possibilities I used a CaMV recombinant in which ORFII, encoding the aphid transmission helper protein, has been replaced with the gene for modified GFP and which expresses GFP as a free protein (Turner and Covey, unpublished). Spread of virus through the infected plants was visualized by confocal microscopy. Manual inoculation of plants with CaMV-GFP has proved unsuccessful in previous studies (Love *et al.*, 2006, unpublished); therefore infection was achieved by bombardment with gold particles coated in virus DNA (See Materials and Methods 2.11.1). *aux1-100*, *aux1-7*, *tir1-1*, *tir3-1* and the corresponding wild-types, Col-0 and WS were bombarded with virus DNA using the BioRad PDS 1000 Biolistic Particle Delivery System. Plants were examined from 12 dpi through until 28 dpi. Representative inoculated, expanded and newly emerging leaves (five leaves) were taken and examined for each timepoint. Samples were viewed at two different wavelengths, 505-530nm and 560-615nm, the latter allows discrimination between genuine GFP fluorescence linked to the virus and autofluorescence (a potential consequence of cell death). Results are shown in **Figures 3.7**, **3.8**, and **3.9**.

Local lesions were first observed as chlorotic spots on the bombarded leaves at 12 dpi for the wild-types, Col-0 and WS. With time, the lesions grew in size and the plants developed systemic symptoms, including chlorosis, stunting and vein

clearing. GFP fluorescence corresponded with the visual symptoms. Local lesions comprising patches of fluorescent cells about 20-30 cells across were first visible at 12 dpi and then grew in both size and number (See **Figure 3.7A** and **D**). By 15 dpi, GFP could be seen to spread into the vascular system and appeared to follow the pattern of vein clearing (see **Figure 3.7 B** and **E**). Both of the wild-types showed systemic spread of GFP by 18 dpi (see **Figure 3.7 C** and **F**). GFP fluorescence was visible in the expanding and emerging leaves from 18 dpi onwards, and from this point on, the amount of tissue showing GFP fluorescence increased greatly. GFP fluorescence in systemically infected leaves closely followed the pattern of symptom expression and was generally linked with chlorotic regions.

In *aux1-100*, *aux1-7*, *tir1-1* and *tir2-2* the patterns of GFP expression were indistinguishable from the corresponding wild-type (see **Figure 3.8**). However, in the *tir3-1* mutant visual assessment did not identify any development of chlorotic local lesions earlier than 21 dpi (see **Figure 3.9 E**). Although wild-type plants typically showed 4-6 fluorescent lesions per leaf as early as 12 dpi, in *tir3-1* even by 18dpi there were fewer than 2 lesions per leaf. At 21 dpi, these were significantly smaller than those observed for Col-0 plants at 12dpi, typically half the diameter of wild-type lesions (see **Figure 3.9 A and E**). Both vascular spread, and growth of the lesions were also consistently delayed in this mutant. These results show clearly that in *tir3-1* both systemic and cell to cell movement of the virus is impaired, perhaps as a result of reduced replication of the virus.

All samples were examined at higher magnifications but differences between the mutants were not observed. GFP was generally confined to areas of the leaf showing chlorotic symptoms and cells examined within these regions appeared the same, with fluorescence observed in the cell boundary and the nucleus.

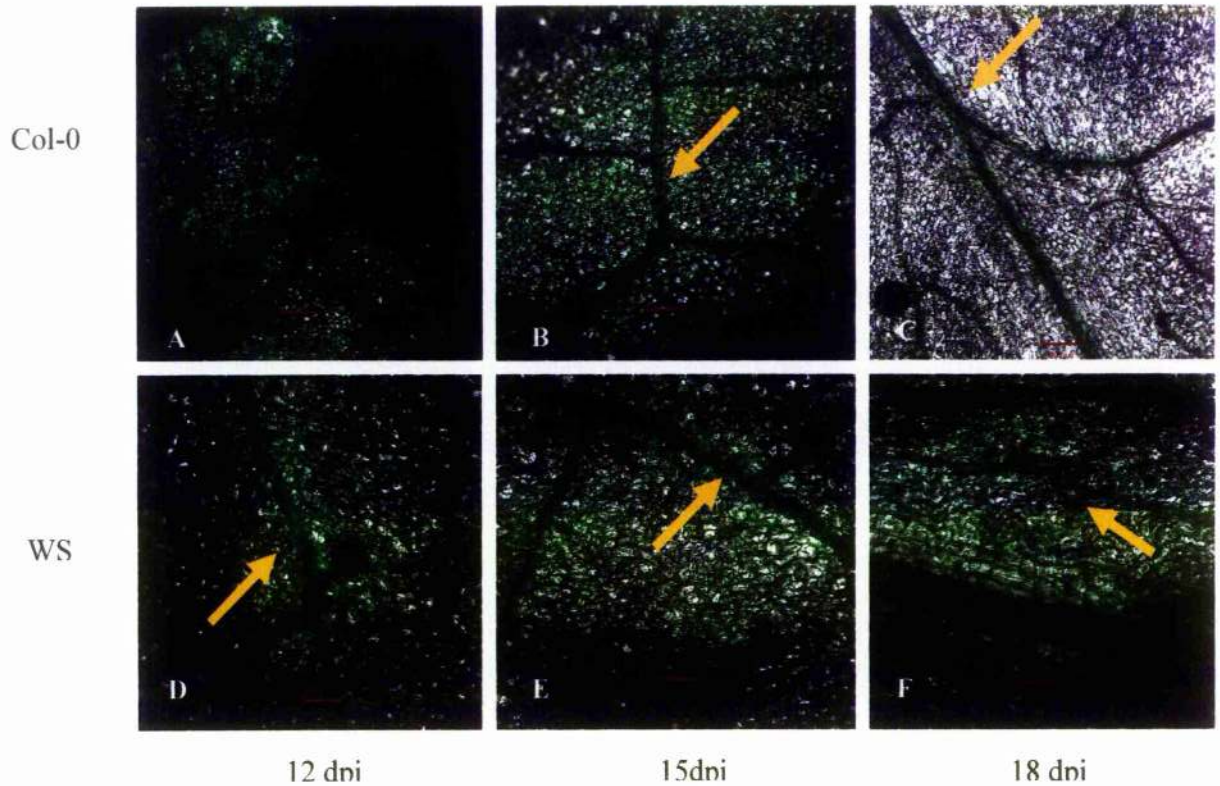


Fig 3.7 Confocal microscope images from wild type, Col-0 and WS infected with CaMV-GFP. All images are at 5x magnification. The excitation wavelength was 488nm and the emission wavelength was 505-530 for GFP. Arrows indicate areas of virus related GFP fluorescence. Yellow Arrows indicate infected vasculature. Scale bars equal 200μm

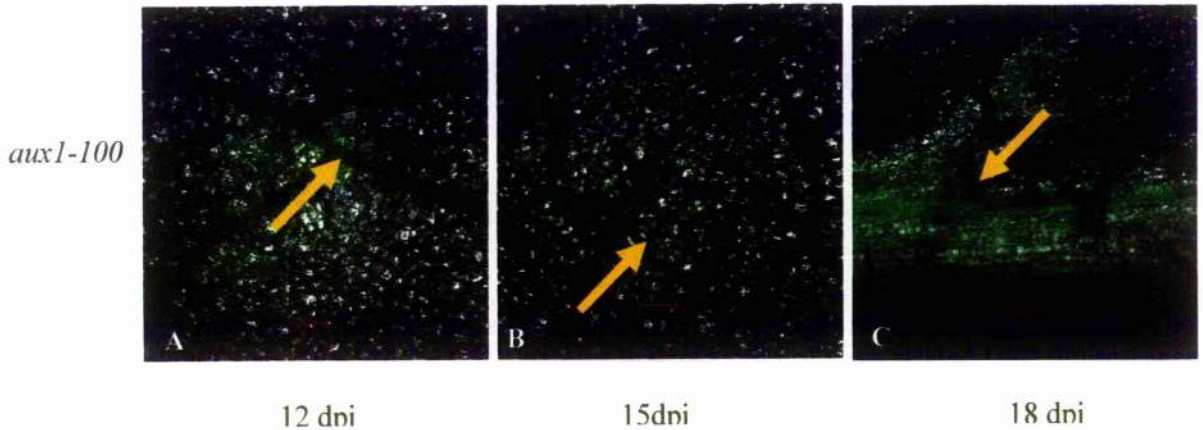


Fig 3.8 Confocal microscope images from *aux1-100* infected with CaMV-GFP. All images are at 5x magnification. The excitation wavelength was 488nm and the emission wavelength was 505-530 for GFP. Arrows indicate areas of virus related GFP fluorescence. Yellow arrows indicate infected vasculature. Scale bars equal 200μm

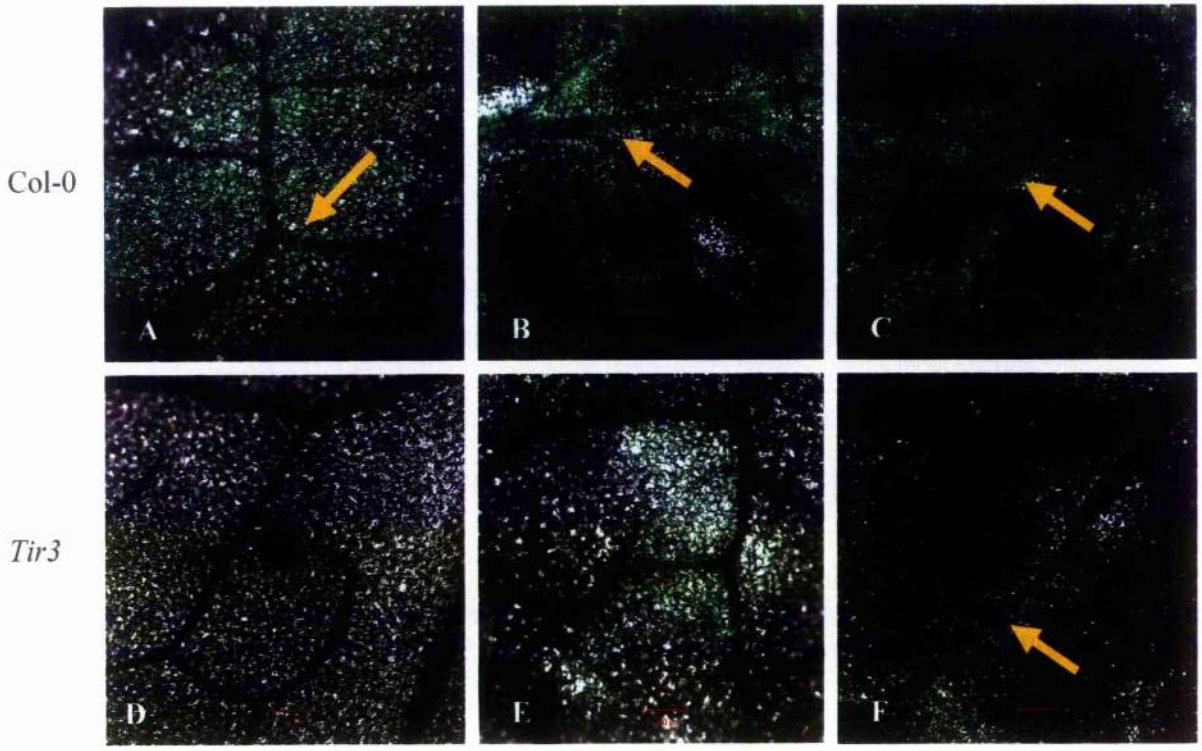


Fig 3.9 Confocal microscope images from plants infected with CaMV-GFP. All images are at 5x magnification. The excitation wavelength was 488nm and the emission wavelength was 505-530 for GFP. Arrows indicate areas of virus related GFP fluorescence. Yellow arrows indicate infected vasculature. Scale bars equal 200µm

Summary (3.3)

These results demonstrate a link between at least one component of the auxin signalling and transport pathway and CaMV infection. A significant difference in symptom response, virus accumulation and spread between a mutant *tir3*, which is affected in auxin efflux was observed compared to wild-type. None of the other mutants examined showed any significant alterations in their response compared to the corresponding wild-types. Although, it is important to note that differences in response to infection occur between the wild-types Col-0 and WS. WS appears to have a less stunted phenotype and develops systemic symptoms slightly earlier than that of Col-0. In *tir3-1,* virus accumulation and spread were reduced and delayed. This delay corresponded with the delay already observed in the development of symptoms and the two are presumably related. Two other mutants, *cpr5* (constitutive expression of PR genes 5) identified from a screen for constituitive expression of systemic acquired resistance (SAR) and *etr1* (ethylene response 1) also show reduced virus accumulation. However in these, unlike in *tir3-1*, this is largely a result of reduced long-distance virus movement (Love *et al.*, 2006, unpublished). The *aux1* alleles and *tir1-1* and *tir2-2* did not show any significant differences compared to wild-type. Since other mutations affecting PAT (*aux1*) or auxin signalling (*tir1*, *tir2*) did not significantly affect CaMV accumulation, the role of *TIR3* may be fairly specific. The role of *TIR3* in vesicle trafficking may be of importance and account for the auxin responses observed, rather than a direct involvement of auxin. This could help explain why the other auxin mutants failed to show any differences in response to infection.

Chapter Four

The effect of CaMV Infection on Virus Induced Gene expression

Introduction (4.1)

Response to virus infection within host plants induces alterations in the expression patterns of a variety of host genes involved in a wide range of processes within the host plant. For example, Whitham *et al.*, (2003) demonstrated that infection by five different RNA viruses elicited co-ordinated changes in gene expression in Arabidopsis plants. These included alterations in the expression of genes associated with distinct defence and stress responses. Geri *et al.*, (1999) showed through the use of differential display PCR that the expressions of various Arabidopsis genes were either up or down regulated by CaMV infection and also by transgenic expression of the viral pathogenicity determinant P6. Microarray investigations have identified a variety of Arabidopsis genes altered in response to virus infection (www.genevestigator.ethz.ch).

Preliminary data was obtained from a full genome Affymetrix ATH1 microarray investigating the effects on gene expression of CaMV infection using RNA extracted from CaMV- infected Col-0 plants (Laval V. and Milner J., unpublished). Approximately 1100 genes from Arabidopsis were identified that showed greater than 3-fold changes in expression and of these, seven genes identified from the annotation as being auxin responsive were highly up regulated in infected plants. These results provide preliminary evidence that CaMV infection induces changes in auxin levels, transport or perception (see

Table 4.1). However, the microarray studies provided no direct correlation between the symptoms or changes in host gene expression during infection, and the auxin levels, concentration and/or distribution. To do so more quantitative and accurate techniques along with a molecular genetic approach are required.

The objective of the work described in this chapter was to examine the effect that CaMV infection has on auxin regulated gene expression and auxin levels during infection.

Control	Infected	Infected/Control	Description
3.4	268.2	79	Auxin-responsive - like protein Nt-gh3 deduced protein, Nicotiana tabacum, EMBL:AF123503 GH3-like
42.4	1025	24	Putative protein auxin-regulated gene, Vigna radiata SAUR e
105.1	2007.6	19	Coded for by A. thaliana cDNA T46835 similar to several small proteins (~100 aa) that are induced by heat, auxin, ethylene and wounding such as Phaseolus aureus indole-3-acetic acid induced protein ARG (SW:32292); supported by full-length cDNA: Ceres:23194. SAG21
19.4	124.8	6	Auxin conjugate hydrolase (ILL5) identical to auxin conjugate hydrolase [Arabidopsis thaliana] (ILL5) GI:5725649;contains nonconsensus AT acceptor splice site at exon3
22.6	76.3	3	Auxin-induced protein IAA5, putative similar to auxin-induced protein IAA5 GI:972913 from Arabidopsis thaliana
75.2	229.2	3	Auxin-inducible gene (IAA2) identical to auxin-inducible gene (IAA2) GB:AF027157 [Arabidopsis thaliana] (Plant Physiol. 115, 1730 (1997))
23.3	69.7	3	Unknown protein contains similarity to auxin-induced protein GB:P33082 from [Glycine max]

Table 4.1. Details of 7 genes from an Affymetrix ATH1 full genome chip which showed greater than 3-fold increases in expression levels and annotated as being auxin responsive.

Results (4.2)

Expression patterns of auxin responsive genes during CaMV infection (4.2.1)

To further analyse how the expression of putative auxin-responsive genes was affected by CaMV infection, a full timecourse of expression of the three most highly up-regulated genes identified from the microarrays was carried out (See **Table 4.2**). Wild-type Arabidopsis and the auxin mutants discussed in Chapter 3 were infected with the severe isolate of CaMV, Cabb B-JI. The *aux1* and *tir* mutants were used as it was thought that if auxin transport or signalling is involved during infection then disruption in these pathways should lead to altered responses to infection. Tissue samples were taken in triplicate during the infection process at timepoints from 0 dpi through until 28 dpi. Total RNA was extracted from each of the samples (see Materials and Methods, 2.6.1) and the expression levels quantified using quantitative slot-blot hybridisation.

Gene	Description	Reference
SAG21 (At4g02380)	Encodes late embryogenesis abundant protein, mRNA levels are elevated in response to various stresses including cold, and reactive oxygen species.	Weaver *et al* (1998)
SAUR_e (At4g34750)	Auxin-induced protein, contains similarity to IAA induced protein ARG7 from *Phaseolus aureus*.	Reddy *et al* (2002)
GH3-like (At5g13320)	auxin-responsive-like protein, Nt-gh3 deduced protein, *Nicotiana tabacum*.	Hagen *et al*., (1991) Guilfoyle *et al*.,(1993)

Table 4.2 Auxin responsive genes identified from microarray investigations as being highly up-regulated in response to CaMV infection.

Total RNA was extracted and treated with DNase to remove any contamination with genomic DNA. The RNA was then added directly onto the membrane by vacuum blotting and once dry, stained with toluidine blue stain to allow the total amount of RNA loaded to be visualised and quantified (See Materials and Methods 2.6.4). The stained membrane was scanned using a flatbed scanner and the RNA quantified using QuantiScan as described by Cecchini *et al*., (2002). The filters were then probed with the gene of choice (See Materials and Methods 2.7) and any radiolabelled probe that hybridised was detected using a phosphorimager. Bands were quantified using QuantiScan and the results normalised against initial RNA quantities (Cecchini *et al*., 2002).

The results are shown in **Figures 4.1, 4.2** and **4.3**, because *aux1-100* is in a WS background and *aux1-7* and *aux1-22* are in a Col-0 background these have been

shown separately. In Col-0 and WS (see **Figures 4.1**) levels of expression were very low or undetectable for all three genes during the early stages of infection, 0dpi to 8dpi, after 8dpi levels increased rapidly. All three *aux1* alleles and the corresponding wild-types (Col-0 and WS) showed an increase in the expression of the selected auxin responsive genes over the duration of the timecourse, which was concomitant with virus accumulation. Both *aux1-7* and *aux1-22* showed patterns of expression (see **Figure 4.2**) similar to that of their wild-type, Col-0 for all three genes examined. ANOVA of the data indicated that there were no significant differences ($p>0.05$) between levels of expression of the

three genes between the mutants and wild-types. *aux1-100* also showed early low level expression followed by a rapid increase in transcript levels from 8 dpi onwards but from 12 dpi on transcript levels were greatly reduced in comparison with the wild-type Ws (see **Figure 4.3**). For all three genes studied, *GH3*-like, *SAUR_e* and *SAG21*, ANOVA indicated a significant reduction ($p<0.05$) in transcript levels in *aux1-100* infected plants compared to the corresponding wild-type.

These results show that CaMV infection is accompanied by an increase in the transcript levels of three of the auxin responsive genes and confirm the microarray results indicating that the expression of these auxin-responsive genes is greatly stimulated by infection. This virus dependent rise in transcript levels is significantly lower in *aux1-100*, a complete null mutant that does not produce

any functional protein, but not in *aux1-7* and *aux1-22* which contain mutations and produce altered proteins with partial function.

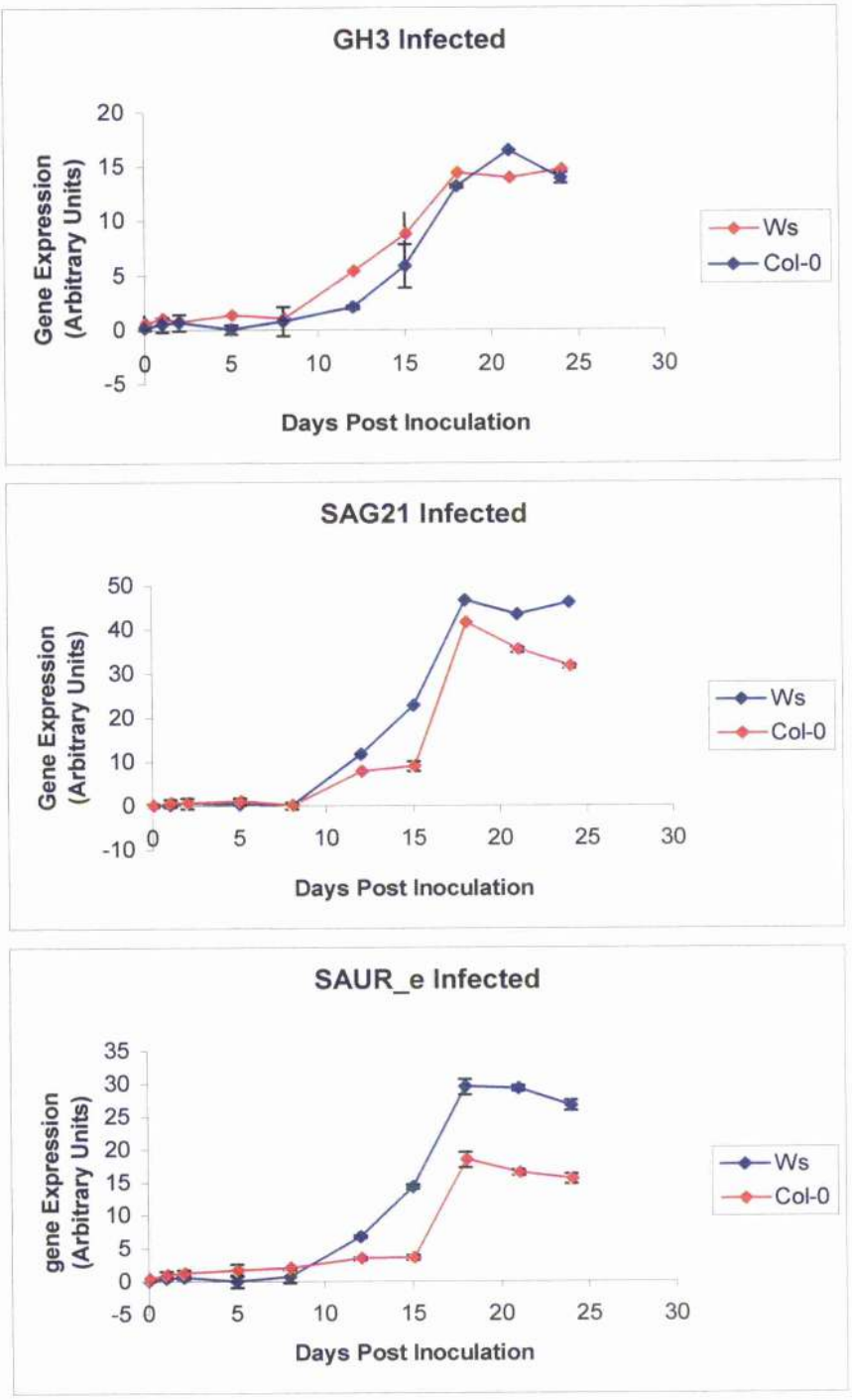


Fig 4.1 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in wild-type Col-0 and WS as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

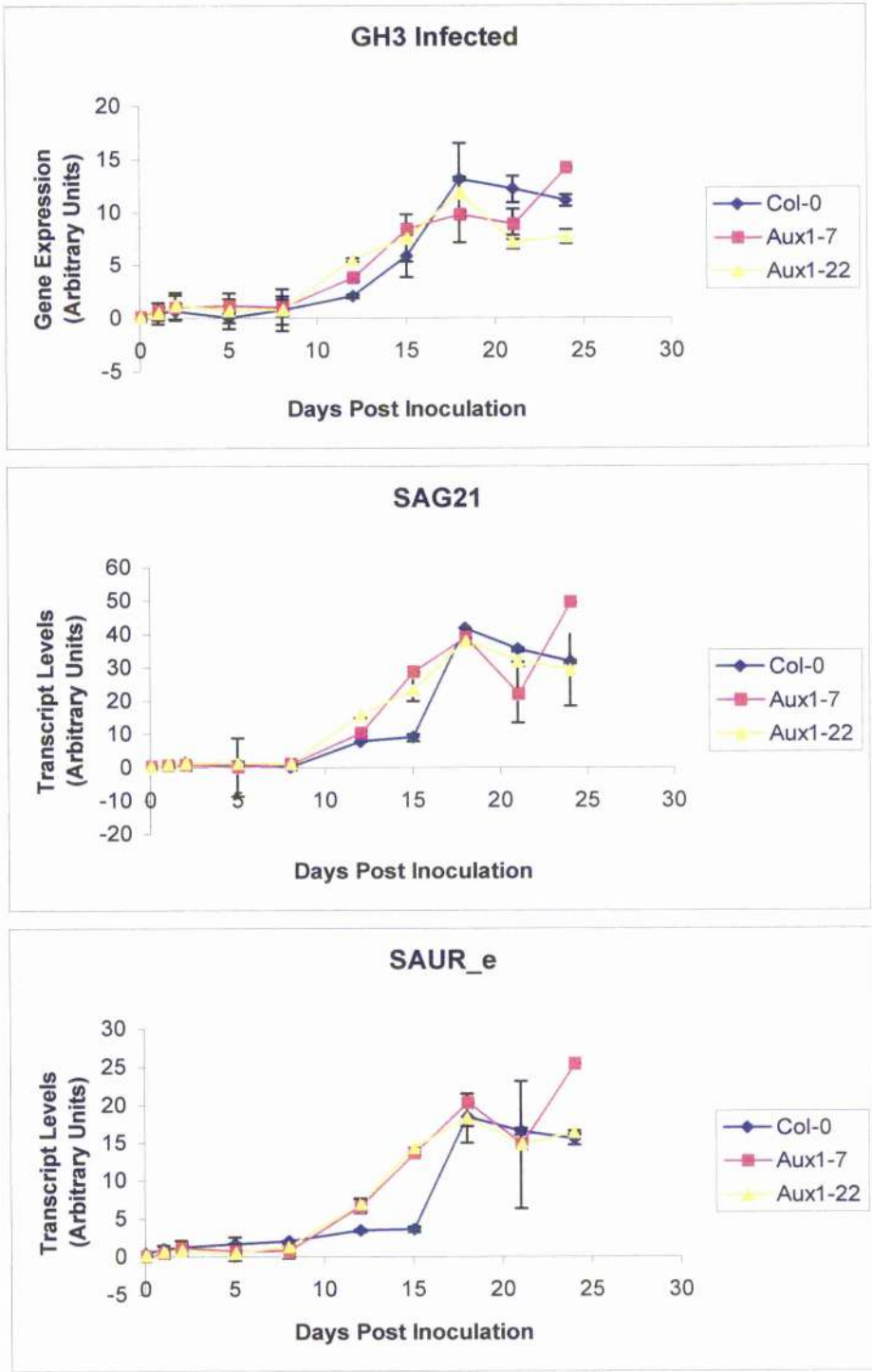


Fig 4.2 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in *aux1-7*and *aux1-22* and the respective wild-type Col-0 as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

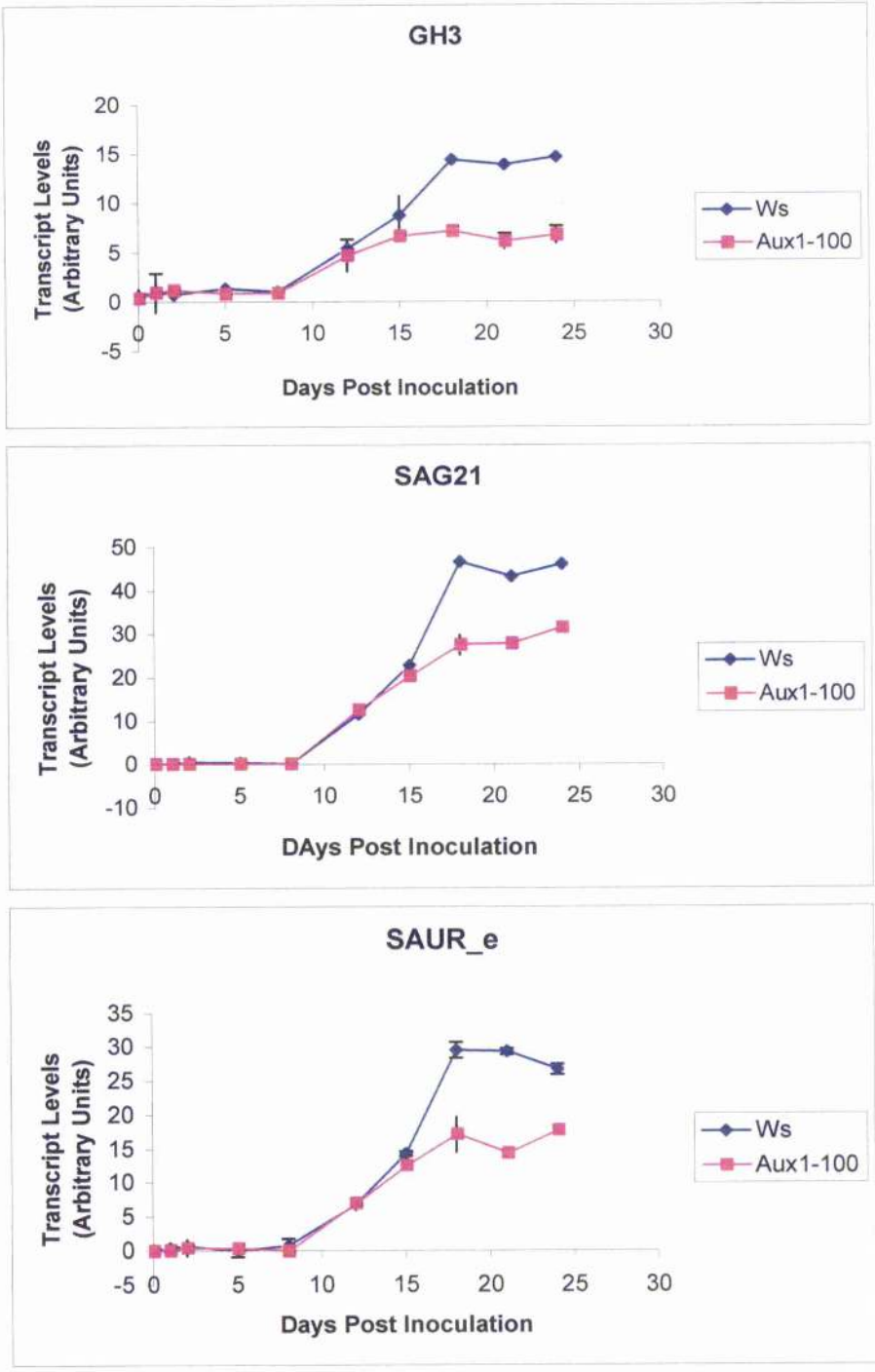


Fig.4.3 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in *aux1-100* and the respective wild-type WS as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

Expressions of these three genes were then examined in the three *tir* mutant lines, *tir1-1*, *tir2-2* and *tir3-1* (see **Figure 4.4**). *tir1-1* and *tir2-2* showed a similar pattern of expression to Col-0 for all three genes, *SAG21*, *GH3*-like and *SAUR_e*, and were not found to be significantly different throughout the course of the infection from the wild-type. In *tir3-1*, *SAG21* and *GH3* levels were similar to those of Col-0 and ANOVA showed that there were no significant differences ($p>0.05$). However, in *tir3-1* the virus dependent increase in *SAUR_e* transcript levels was lower than in wild-type. Indeed, in *tir3-1* levels of *SAUR_e* transcripts at 21 dpi were not significantly greater from those at 0dpi. These results suggest that *TIR3* may be required for virus-induced up regulation of *SAUR_e* but not *SAG21* or *GH3*.

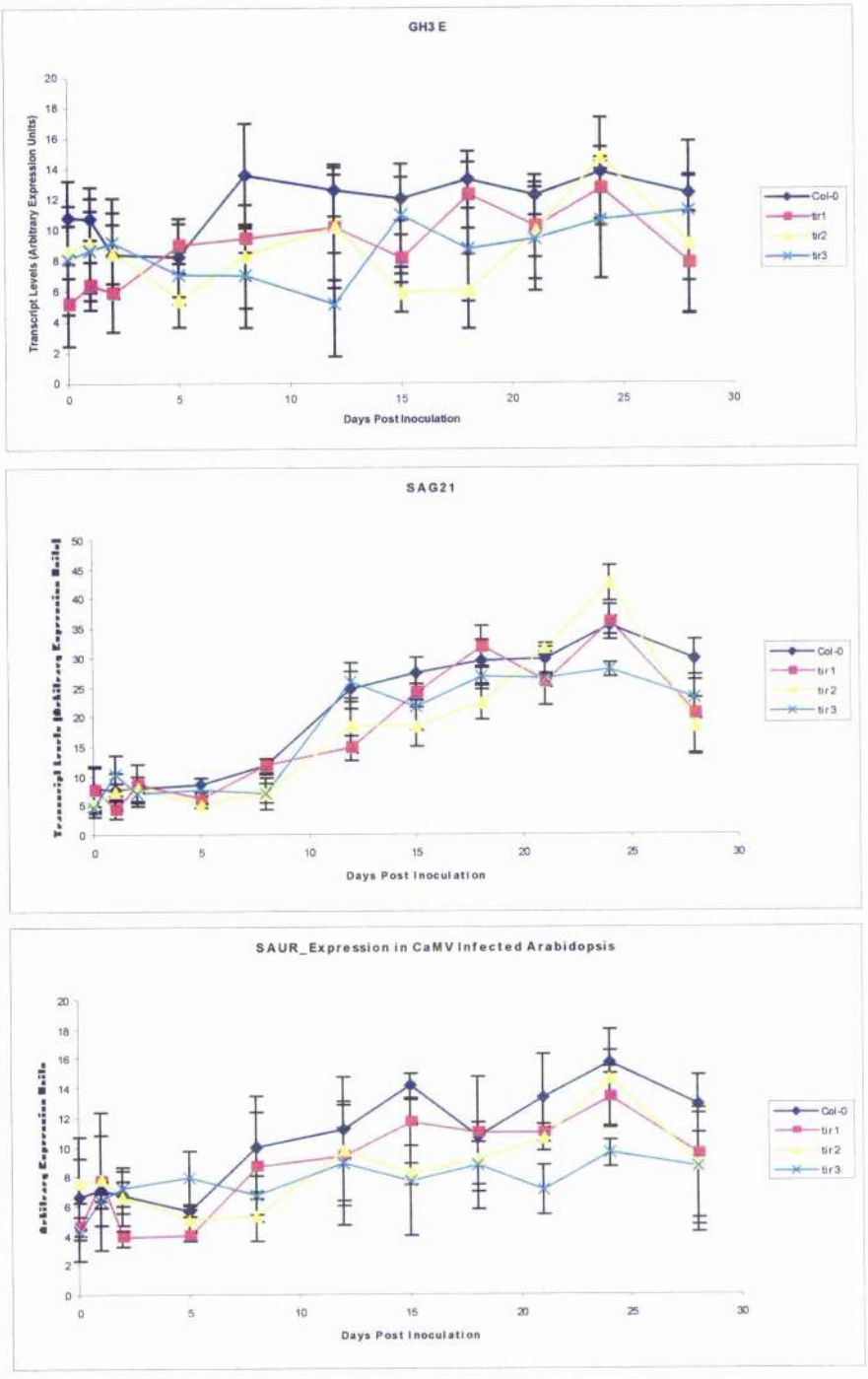


Fig 4 .4 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in *tir1-1*, *tir2-2* and *tir3-1* and the respective wild-type Col-0 as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

The effect of CaMV on the Expression Of Further Auxin Regulated Genes using Reporter Promoter Fusions (4.2.2)

To examine further the effect of CAMV on the expression patterns of auxin-responsive genes, transgenic plants containing an auxin-responsive promoter fused with a GUS reporter gene were examined. *LAX2* is an *AUX1* analogue that is involved in the loading of auxin via the phloem, and expression of *LAX2* is auxin responsive (Bennett, unpublished; Parry *et al.*, 2001). This gene is expressed mainly in the shoot tissue, and as this is the principle site of virus replication it should provide a useful marker for any dependent auxin response. Plants were infected with Cabb B-J1 at the two true leaf stage (see Materials and Methods 2.3.3) harvested at suitable times after inoculation and stained for GUS activity. To distinguish between virus and wounding responses mock-inoculated plants were used as a control. Results are shown in **Figure 4.5**.

In mock-inoculated plants at the very early stages after mock inoculation, GUS staining was limited to the apical region in the centre of the seedling; no GUS stain was seen in the surrounding shoot tissue. By 14 dpi, at which point the infection had progressed and symptoms had begun to appear in virus-inoculated plants, GUS staining could be seen in the surrounding leaf tissue. The area of GUS staining continued to expand and by 28 dpi the mock inoculated samples showed extensive staining of the rosette leaves. Virus-infected plants showed a similar pattern of staining to the mock-inoculated plants at the early stages of the infection process; GUS stain was limited to the apex and no GUS was observed in the surrounding leaf tissue. As the infection progressed and symptoms became

visible, GUS staining remained confined to the plant apex, in contrast to the expanding area of stain in mock-inoculated plants. Even by 28 dpi GUS was still confined to the apex whereas mock-inoculated controls showed extensive staining at the same stage. These results demonstrate that in uninfected plants there is a developmental increase in *LAX2* expression. This effect, which may reflect changes in auxin distribution or signalling, appears to be suppressed by CaMV infection since the expansion of the stained area was absent in virus-infected plants.

DR5 is a synthetic auxin response element, consisting of seven tandem repeats of the AuxRE TGTCTC motif (Mattsson *et al.*, 2003; Ulmasov *et al.*, 1997). DR5::Reporter fusions have been used as a tool to trace changes in auxin distribution (Mattsson *et al.*, 2003). Plants were either mock inoculated or inoculated with CaMV isolate Cabb B-JI, and samples examined at timepoints from 0 dpi through to 28 dpi. With DR5::GUS both mock inoculated and CaMV infected samples showed uniform GUS staining throughout the leaf tissue at all timepoints studied (see **Figure 4.6**). No obvious changes were observed in either the intensity or distribution of GUS, nor were there any differences between mock-inoculated and virus-inoculated plants. These results suggest either that auxin levels are unaffected by virus infection or that this reporter does not provide a reliable indication of changes in auxin levels.

Confocal microscopy was used to try to visualise alteration in auxin levels using a DR5::GFP construct. Plants containing DR5::GFP were inoculated with CaMV

Cabb B-JI, controls were mock inoculated with water, and the aerial tissue removed and examined over a timecourse, from 0 dpi to 28 dpi. However, no GFP fluorescence could be seen in any of the shoot tissue examined. Data for this is not shown.

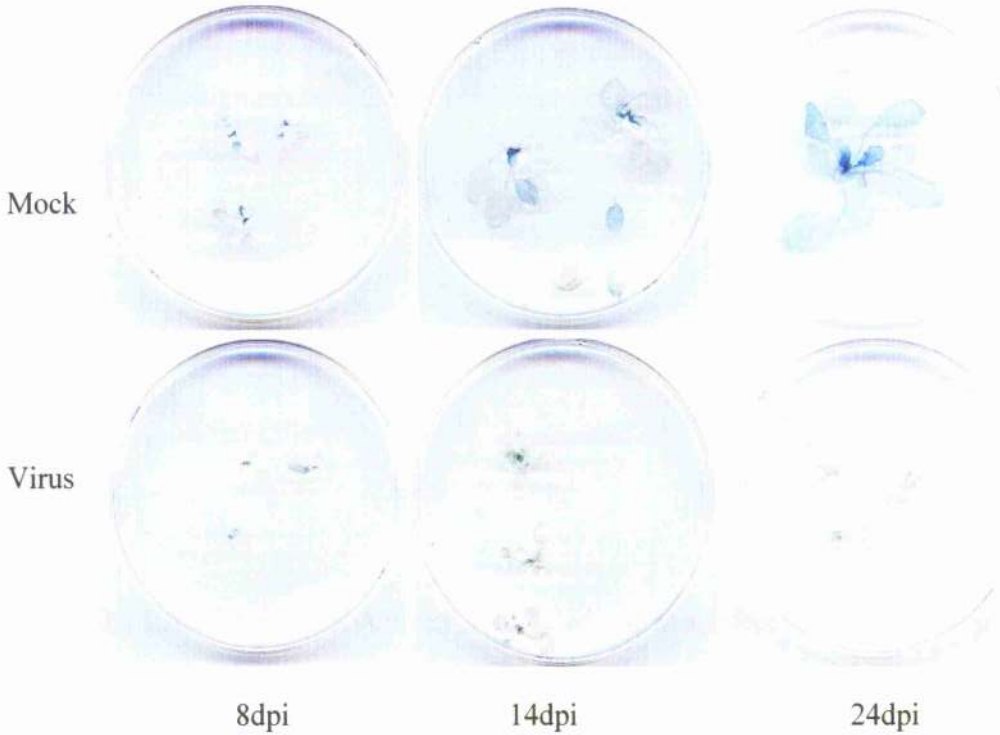


Fig. 4.5 GUS reporter driven by LAX2 promoter. Activity in infected and non-infected LAX2::GUS plants over a timecourse of 24 dpi.

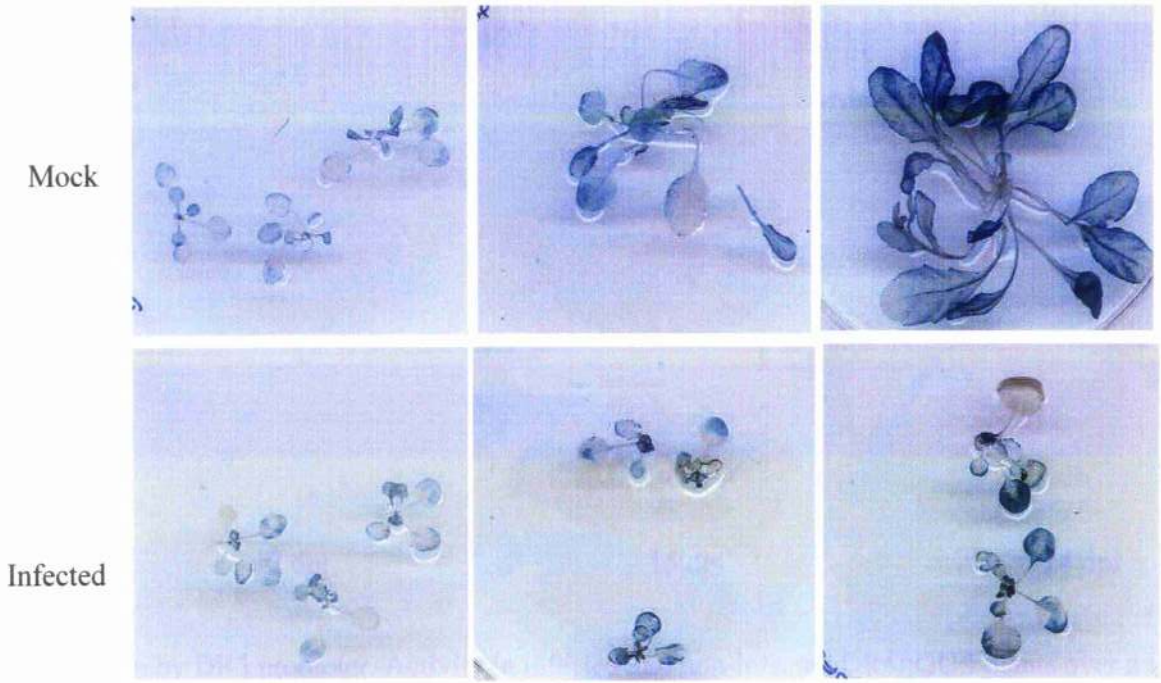


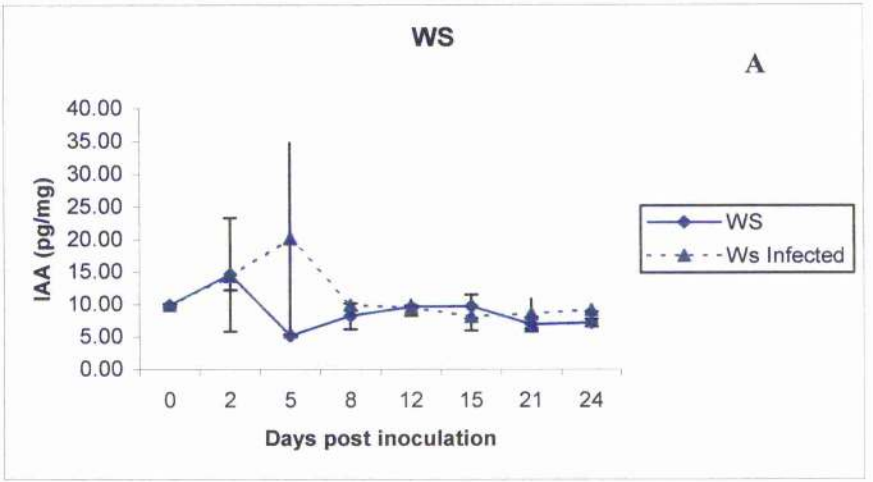
Fig. 4.6 GUS reporter driven by DR5 promoter. Activity in infected and non-infected DR5::GUS plants over a timecourse of 24 dpi.

IAA Analysis and Quantification (4.2.3)

Expression studies on three auxin responsive genes has shown them to be highly up-regulated in response to virus infection. These results suggest that infection may result in alterations in auxin levels that correspond to increased virus titre and spread. Further evidence for changes in auxin levels are provided by the pattern of expression in *LAX2*::GUS reporter lines, although the results with DR5 driven reporters does not support this. To test whether auxin levels are affected by infection, IAA levels were assayed directly. Total free IAA was quantified by GC-MS in samples consisting of pools of plant tissue from approximately 10-12 plants. Plants were infected with CaMV- Cabb B-JI and then harvested over a timecourse from 0 dpi through until 24 dpi. The entire aerial portion of the plant was removed and ground to a fine powder in liquid nitrogen. Of this ground tissue, approximately 20 mg of frozen tissue was taken and the free IAA extracted and purified using an SPE-based purification method. Once purified IAA was quantified using gas chromatography-mass spectrometry as described in Edlund *et al.*, (1995).

Results are shown in **Figure 4.7, A and B.** In both infected and uninfected plants, levels of free IAA showed no significant changes over the duration of the infection. IAA levels were slightly higher during the early timepoints, 0dpi to 5dpi, followed by a reduction, which continued until the end of the timecourse but these differences are not significant ($p>0.05$). There were also no significant differences between *aux1-100* and wild-type ($p>0.05$; see **Figure 4.7 B**). These results indicate that there are no gross changes in auxin levels either as a result

of development or infection. However by harvesting of the entire aerial portion of the plant, any differences in auxin distribution would not have been apparent. This suggests that virus is not affecting the overall amount of auxin within the plants and so alterations in transcript levels of the auxin-responsive genes do not correspond to changes in auxin levels although it could be affecting the distribution of auxin.



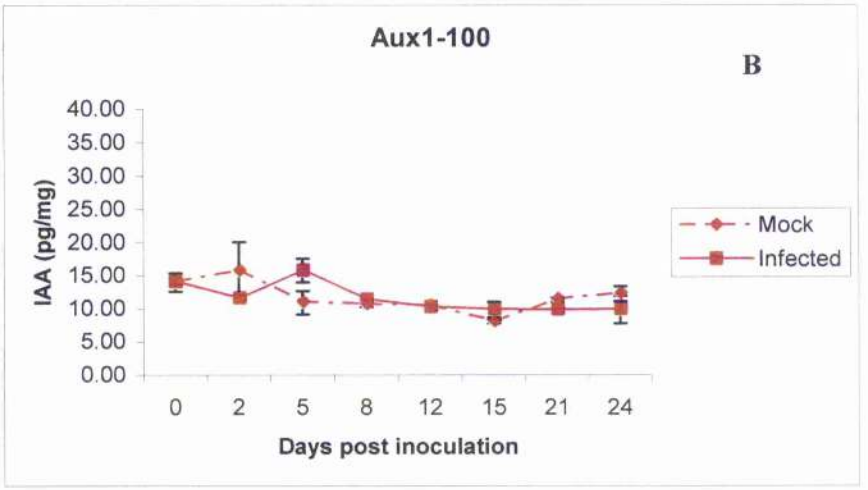


Fig 4.7. Quantification of free IAA from both infected and mock-inoculated plants. Samples were collected from 0 dpi at three day intervals until 24 dpi and IAA extracted and quantified using HPLC techniques. Results are shown with standard errors (n=3)

(A) IAA levels in CaMV infected and mock inoculated wild-type WS.

(B) IAA levels in CaMV infected and mock inoculated *aux1-100*.

Summary (4.3)

The main objective of the work described in this chapter was to define the effect of CaMV infection on auxin regulated responses within the host plant. Microarray studies had identified seven auxin responsive genes that were highly up-regulated in response to infection and here a more complete analysis of the pattern of expression of the three most strongly up-regulated candidate genes was carried out. In three independent experiments virus infection stimulated an increase, up to 8-fold, in the level of *SAG21*, *GH3* and *SAUR_e* transcripts. Transcript levels increased from 8 dpi, this corresponds to the increase in virus titre in these plants (Love *et al.*, 2005). This increase in transcripts was significantly reduced, by about 2-fold, but not abolished in *aux1-100* mutants when compared to WS for all three genes. In contrast the weak alleles *aux1-7* and *aux1-22* showed little difference from wild-type, Col-0. *aux1-7* and *aux1-22* both produce proteins with partial function whereas *aux1-100* is a null mutant and does not produce a functional protein. The significant reduction in responsiveness for all three genes in the *aux1-100* mutant suggests that polar auxin transport facilitates the virus dependent response.

Three transport inhibitor response mutants were compared to wild-type, Col-0. In *tir1-1* and *tir2-2* infection with CaMV levels of *SAG21*, *GH3* and *SAUR_e* transcripts were similar to wild-type. However, *tir3-1* showed a significant reduction ($p<0.05$) in virus dependent *SAUR_e* expression compared with the wild-type, Col-0. The *TIR3* gene product is involved in auxin efflux (Ruegger *et*

al., 1997), whereas *tir1* and *tir2* are affected in auxin signaling. Again this result points to a possible role for polar auxin transport in the virus induced response.

Work from Bennett (unpublished) has shown that *LAX2* expression is auxin-responsive and that the gene is expressed mainly in the shoot tissue. It is an *AUX1* homologue and is believed to be responsible for the loading of IAA into the phloem. Developmental expression of GUS driven by the *LAX2* promoter was almost completely suppressed in infected plants, and GUS expression did not spread into the rosette leaf tissue, but remained confined to the apex. These results provide more evidence to suggest that CaMV infection is affecting auxin regulated gene expression and suggest that some aspect of auxin signaling or transport is affected. However GUS expression driven by the synthetic auxin responsive element DR5 failed to show any alterations in auxin distribution in response to either development or virus infection. This suggests that either auxin is unaffected, or that the DR5::GUS reporter line is not a reliable indicator of auxin levels and distribution. Another explanation for the inconsistencies between these two studies could be due to the expression pattern of the two constructs. LAX2 is expressed in the shoot tissue at points where loading of IAA into the phloem occurs and so will presumably only respond to local changes in auxin concentration or distribution. Whereas DR5 is expressed throughout the plant and therefore it is possible that any small local changes in auxin observed when we look only at LAX2::GUS are not observed when we look at DR5::GUS as this is responding to changes in auxin throughout the plant. It might therefore

be useful to examine these plants at a cellular level to see if there are changes in auxin response between cells that could not be seen during this investigation. DR5 is useful as a global marker of auxin response whereas LAX2 is an effective local marker.

Previous studies have tried to establish the effect that virus infection has on the levels of free IAA within plants and to link the physical symptoms observed during infection to these changes (reviewed by Pennazio and Roggero, 1996). The results have been inconsistent and different studies have shown both increases and decreases in auxin levels depending on the virus and host studied. Here, to establish if CaMV infection was affecting auxin levels within symptomatic plants, GC-MS was used to quantify the levels of free IAA within

both infected and mock inoculated plants. No differences in overall IAA levels between infected and mock inoculated plants were identified either in wild-type or *aux1-100*. This result illustrates the difficulties in establishing interactions between virus infection and auxin signalling by measuring auxin levels in tissue samples. Auxin distribution could be affected but the changes too subtle to be observed with the techniques used here. For example, auxin levels could be altered in areas of chlorosis compared to unaffected areas, between different tissue types or even between cells. Results using auxin-responsive reporters are contradictory. LAX2::GUS suggests a virus dependent change in auxin response, whereas no such change was identified with DR5::GUS. LAX2 is a naturally occurring auxin responsive gene and so the promoter could be responding to

other developmental cues within the plant, whereas DR5 which is completely synthetic will presumably not respond to anything other than auxin. These differences might account for the conflicting results from the two lines.

Chapter Five

Interaction Between CaMV Gene VI and Auxin Signalling and Transport

Introduction (5.1)

The work described in the previous chapters suggests that auxin signalling components are involved in the host response during CaMV infection. One possibility may be that CaMV gene products interact with components of auxin signalling or distribution. A possible candidate for this interaction is the CaMV protein P6. P6 functions as a translational transactivator, regulating the expression of other CaMV genes through modification of the host translation machinery (Reviewed in Chapter 1). As well as this role it is also the major genetic determinant of pathogenicity (Reviewed in Chapter 1). Recombinant virus genomes produced from CaMV isolates with different pathological characteristics have demonstrated that gene VI encodes pathogenicity determinants including those involved in host range and symptom determination (Rothnie *et al.*, 1994; Daubert *et al.*, 1990). Work from Hohn's group suggests that different domains may be involved in translational transactivation and pathogenicity (Kobayashi and Hohn, 2003; Kobayashi and Hohn, 2004). In the absence of virus infection in Arabidopsis and tobacco, P6 transgenic plants can show a symptom-like response (Cecchini *et al.*, 1997; Baughman *et al.*, 1988). P6 transgenic Arabidopsis plants show the symptoms commonly associated with CaMV infection, including vein clearing, chlorosis and stunting (Cecchini *et al.*, 1997). The type and severity of the symptoms observed correlated with the severity of the isolate from which the P6 was obtained and also to the levels of

P6 expression within the transgenic plant (Cecchini *et al.*, 1997). Most likely, during infection the host response is dependent not only on the levels of virus present but also on interactions between the CaMV and host-encoded components, triggering a set of pre-programmed responses. A likely candidate for this interaction is P6 as it appears to play a critical role in the host response. P6 transgenic Arabidopsis plants show many of the changes in gene expression that are detected in infected plants (Geri *et al.*, 1999). Therefore P6 appears to be accountable for these changes.

Previous studies have shown that P6 interacts with ethylene signalling during the infection process (Geri *et al.*, 2004), and P6 transgenic plants display an ethylene insensitive phenotype. P6 has also been shown to be a potent suppressor of SA-mediated defence responses (Love *et al.*, paper in preparation). Preliminary investigations into the effect of 1-naphthylphthalamic acid (NPA), an auxin inhibitor suggested that the symptom-like phenotype of P6 transgenics was ameliorated reversibly when grown on NPA, suggesting a link between auxin signalling and P6 (Cecchini and Milner, unpublished observation). The objective of the work described in this chapter was to establish whether CaMV P6 interacts with components of the auxin signalling and transport pathways, and if so whether this could account for the apparent auxin dependent responses reported in chapter 4. The P6 transgenic plants are a valuable resource with which to investigate this question.

P6 Expression Levels in P6 Transgenic Arabidopsis (5.2.1)

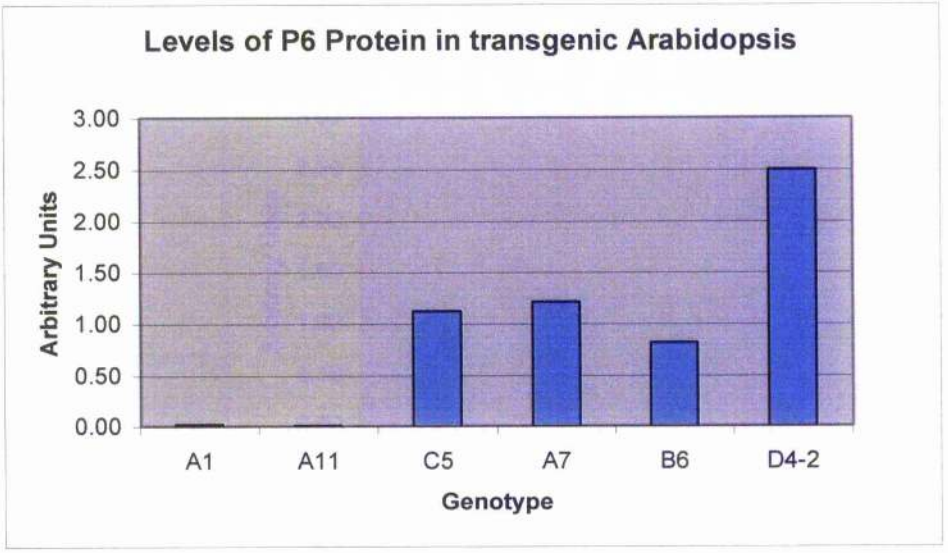
Cecchini *et al.*, (1997) constructed more than 40 transgenic lines that express P6 from different CaMV isolates in an Arabidopsis Ler-*gl1* background. Levels of P6 protein in these lines were originally assayed in T2 plants, because transgene expression can change from generation to generation as a result of silencing, protein levels were assayed in the T6 plants used for these studies. P6 protein levels were also assayed in line D4-2 (a gift from J. Schoelz). The D4-2 transgenic line is homozygous for gene VI from the D4 isolate of CaMV (Yu *et al.*, 2003). In contrast to Cabb B-JI the D4 strain of CaMV induces only mild symptoms in infected plants. Transgenic Arabidopsis expressing gene VI from D4 were almost symptomless and appeared similar to wild type. To establish directly the levels of P6 protein in the P6 transgenic Arabidopsis lines, western blotting was used to quantify the protein levels present in T6 plants. P6 protein was detected using a polyclonal antibody raised against P6 from CaMV strain W260 (Schoelz *et al.*, 1991). This antibody has been shown to cross react with P6 from a number of CaMV strains including D4 (Yu *et al.*, 2003), Cabb B-JI (Milner, unpublished data). Five independent transgenic lines were examined, A1, A11, C5, A7, B6 (which all containing a gene VI construct derived from Cabb B-JI; Cecchini *et al* 1997) and D4-2 (see Table 5.1). Protein was extracted from samples of each transgenic line (see Materials and Methods, 2.12), western blots carried out and probed and the protein levels quantified using Quantiscan (as described for RNA).

Transgenic Line	CaMV Isolate	Phenotype	P6 Transcript Levels (Cecchini *et al.*, 1997)
A7	Cabb B-JI	Stunted, chlorotic	High
B6	Cabb B-JI	Stunted, chlorotic	High
C5	Cabb B-JI	Stunted, chlorotic	Moderate
A1	Cabb B-JI	Nontransgenic phenotype	Low
A11	Cabb B-JI	Nontransgenic phenotype	Undetectable
D4-2	D4	Mild chlorosis, no stunting	High

Table 5.1 Phenotypes and P6 mRNA levels in T2 transgenic Arabidopsis plants expressing Cabb B-JI gene VI driven by the 35S promoter as determined by northern blots (Cecchini *et al.*, 1997).

The transgenic lines were tested in western blots using the polyclonal rabbit anti-P6 antibody. Two silenced lines, A1 and A11, were used as controls. These have been shown previously to have low levels of P6 mRNA (Cecchini *et al.*, 1997). Western blots confirmed this as P6 protein was undetectable in these lines. C5, B6 and A7 all contained significant levels of P6 protein. In contrast to T2 plants where C5 was reported to have only moderate P6 mRNA levels, P6 levels in C5 were similar to A7 and higher than in B6. In D4-2, which contains gene VI from the D4 isolate of the virus, signals in the Western blot were about twice that from A7. Both A7 and B6 show a strong phenotype, including stunting and chlorosis. The D4-2 transgenic line appears phenotypically wild type despite accumulating greater levels of P6 than the severely chlorotic and stunted A7 and B6. However, it is possible that differences in the cross reactivity of the antibody could account in part for the apparent high P6 levels in D4-2.

A



B

Fig 5.1 Quantification of P6 protein in P6 transgenic Arabidopsis plants. (A). Photograph of western blot showing P6 protein, detected using chemiluminescence (B) Quantification of levels of chemiluminescence from western blots using Quantiscan (as described for RNA).

Interaction Between CaMV Protein P6 and Auxin Signalling and Transport (5.2.2)

P6 transgenics which express gene VI from the Cabb B-JI isolate of the virus were reported to show an ameliorated reversible phenotype when treated with NPA, an auxin analogue that can also act as an inhibitor of auxin transport (Cecchini and Milner, unpublished). However the levels of NPA used in these studies were very high. Therefore a different inhibitor of auxin efflux was tested. Plants were grown on a series of increasing concentrations of 2,3,5-triiodobenzoic acid (TIBA), an inhibitor of carrier-mediated auxin efflux. TIBA may block auxin efflux by competitively binding to the auxin binding protein (Rubery, 19990; Muday and DeLong, 2001). The mechanism of action of this compound is not completely understood, but it may either directly block auxin movements or indirectly alter the cycling of auxin transport proteins to or from the plasma membrane (Muday and DeLong, 2001). Both the phenotypic response and the expression levels of an auxin responsive marker gene, *SAUR_e* were examined at 16 and 24 days after germination. The phenotypes were assessed visually and plants were photographed. Levels of *SAUR_e* transcripts were determined using RT-QPCR (See Materials and Methods, 2.9). *SAUR_e*, which is an auxin responsive gene, is a member of the small auxin up-regulated RNA protein family. Results in Chapter 4 found this gene to be highly up-regulated in response to CaMV infection. Three independent transgenic lines were used, A7 and B6, which express high levels of P6 from isolate Cabb B-JI and D4-2, which contains P6 from the D4 isolate. *aux1-100* was used as a control. Also tested were P6 lines, B2-3 (*cse-2*) and

B4-2 (*cse-1*). These lines, which carry, a suppressor mutation, *cse* (CAMV P6 Suppressor-Ethylene Insensitive), were identified from a screen for mutants that suppressed the P6 phenotype of chlorosis and stunting. Seeds from the transgenic P6 line A7 were mutagenized by γ-irradiation and M2 seedlings were screened for mutants that showed a reduced P6 phenotype. Two lines, B2-3 and B4-2, that are allelic for recessive mutations at the same locus, appear considerably larger and greener than the A7 parental line but have been shown to contain an intact and transcriptionally active transgene. However these plants still show some stunting and chlorosis compared to non-transgenic Arabidopsis. The *cse* mutation does not suppress ethylene or SA insensitivity in P6 transgenics (Geri *et al.*, 2004).

Sensitivity of P6 transgenic plants to TIBA (5.2.2.1)

The transgenic lines together with the *aux1-100* control and wild types were germinated on plates containing TIBA at concentrations, ranging from 0μM to 35μM. Although Geldner *et al.*, 2003 has suggested that concentration above 25 μm may cause cytoplasmic acidification other studies using TIBA to examine the effects of auxin transport on growth of the plant have used a range of concentrations up to100 μm (Chen *et al.*, 2007) or 200 μm (Garcia-Gomez *et al.*, 1994).After 24 days of growth on the plates, phenotypes were assessed and the plants were photographed. The wild type plants, Col-0 and Ler *gl1* developed normally at low concentrations of TIBA, 0μM and 15μM but were unable to grow successfully at concentrations greater than 20μm (see **Figure 5.2 and 5.3**). At concentrations above 20μM, the seedlings appeared stunted

and severely chlorotic. *aux1-100* plants were able to grow and develop normally at concentrations up to 30μM; however at 35μM plants were starting to show stunting and chlorosis. In contrast A7 transgenic plants did not show this sensitivity to TIBA and were able to grow successfully on concentrations of TIBA as high as 35μM. At this concentration the plants appeared similar in appearance to those grown on plates without TIBA added. The seedlings showed no additional chlorosis over and above that associated with the P6 phenotype. The B6 transgenic line was also able to tolerate TIBA concentration up to 35μM. However, it did not grow as successfully as A7 and at 35μM TIBA some plants showed the stunting and severe chlorosis observed in the wild type.

D4-2 also demonstrated enhanced TIBA resistance compared to wild type and plants were able to grow successfully at concentrations of 35μM without any visible stunting or chlorosis. D4-2 showed a strong TIBA-resistant phenotype that appears to be independent of the stunting and chlorosis phenotype induced by expression of P6 from Cabb B-JI but not seen in the D4-2 transgenic line. To test this further the two suppressor lines B2-3 (*cse-2*) and B4-2 (*cse-2*) were tested. The A7 transgenics containing the suppressor mutation, B2-3 and B4-2, showed much reduced resistance to TIBA compared to A7 or B6, and were only slightly more resistant than the Ler *gl1* wild type parent. B2-3 and B4-2 were unable to grow on concentration greater than 20μM; at higher concentrations the plants began to show severe chlorosis and stunted growth.

Fig 5.2 Growth of *Arabidopsis* P6 transgenics and ler gl1 wild-type in response to various concentrations of the auxin efflux inhibitor TIBA.

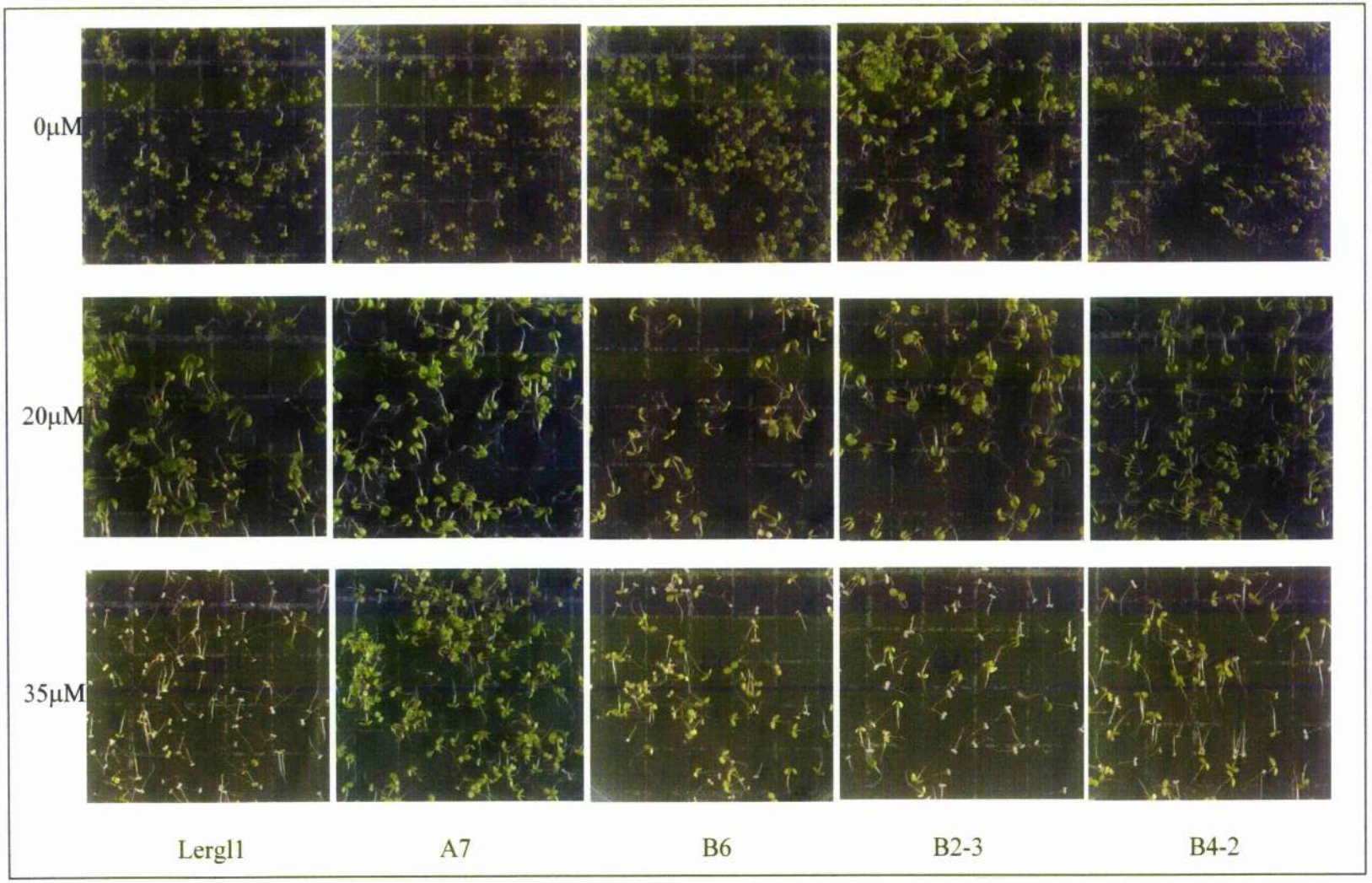
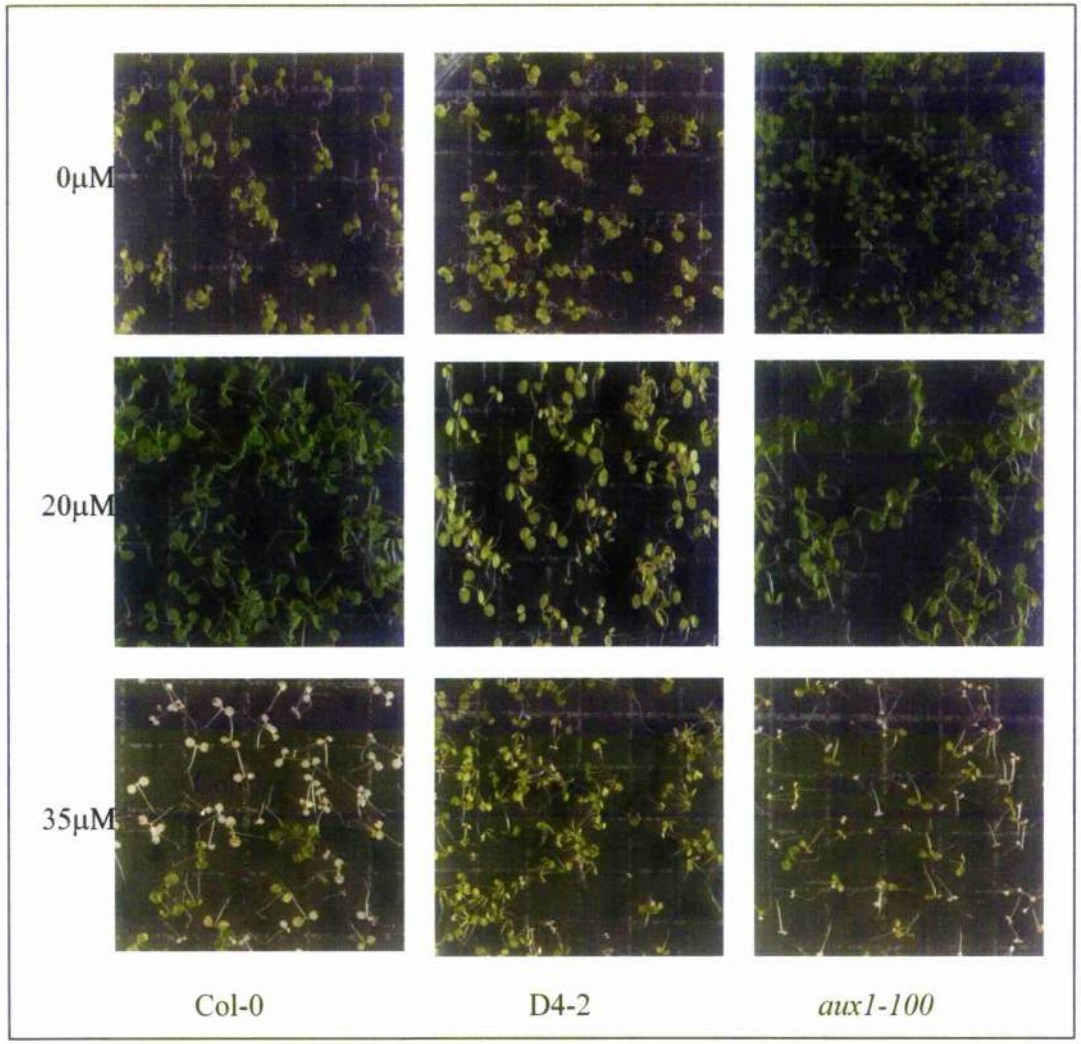

0μM
20μM
35μM
Lergl1
A7
B6
B2-3
B4-2

Fig 5.3 Growth of Arabidopsis P6 transgenics and Col-0 wild-type in response to various concentrations of the auxin efflux inhibitor TIBA.


0μM
20μM
35μM
Col-0
D4-2
aux1-100

Expression of Auxin Responsive Genes in P6-transgenic plants (5.2.2.2)

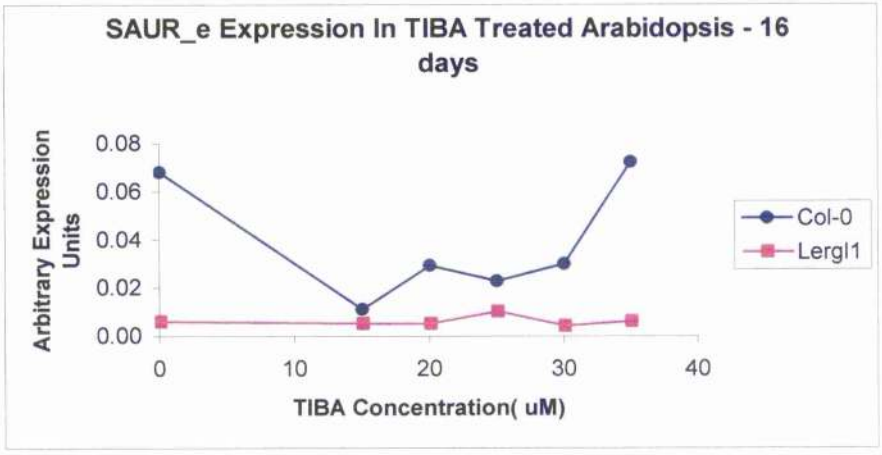
To further analyse the effect of TIBA on the P6 transgenic lines, the expression of an auxin responsive gene was investigated. Infection studies, described in Chapter 4, demonstrated that the auxin responsive gene *SAUR_e* was highly up-regulated in response to CaMV infection. This gene has also been identified as being TIBA-responsive from analysis of Genevestigator and NASC (Zimmermann *et al.*, 2004; Riken Group, 2004, unpublished; genevestigator chip # 113). Experiments carried out in triplicate where wild type seedlings were treated with TIBA found that expression of *SAUR_e* is reduced in response to TIBA treatment.

Plants were grown on plates containing increasing levels of TIBA, from 0-35 μM, and tissue samples were taken at 16 and 24 days after germination. Levels of *SAUR_e* mRNA, were quantified in samples of each of the transgenic lines, *aux1-100* control, and wild types. RNA was extracted and DNAse treated before being quantified. cDNA was synthesised and subsequently used in RT-QPCR reactions. *SAUR_e* expression was normalised against the levels of *ACT2* mRNA. *ACT2* has been found to be expressed constitutively in Arabidopsis, and makes a good internal reference standard for RT-QPCR (Laval *et al.*, 2002, Love *et al.*, 2005)

In the wild type plants, Col-0 and Ler, levels of *SAUR_e* transcripts were low at all concentrations of TIBA (see **Figure 5.4 and 5.5**). In contrast to the study reported in Genevestigator (Zimmermann *et al.*, 2004; Riken Group, 2004,

unpublished; genevestigator chip # 113), levels of *SAUR_e* transcripts showed no consistent decrease in plants germinated on TIBA. Also, transcript levels remained relatively constant between the two timepoints. Real time RT-PCR is an exquisitely sensitive technique and the levels of *SAUR_e* transcripts were very low relative to *ACT2* (equivalent to 10 or fewer copies of the mRNA per cell; see Love *et al* 2005, Laval *et al* 2002). Therefore the results carry a large amount of inherent variation and the wild type plants do not appear to show any consistent pattern of expression in response to TIBA. In contrast, in the absence of TIBA, A7 transgenic plants showed highly elevated levels of *SAUR_e* transcripts, about 30-fold higher than in the Ler *gl1* wild type. Moreover *SAUR_e* transcripts appear to be TIBA responsive. Levels increased a further 2-3 fold when treated with levels of TIBA of 15 µM or more. B6 plants also show high levels of *SAUR_e* transcripts compared to the wild type; however these were less elevated than in A7, approximately 10 fold higher than wild type. D4-2 demonstrated the highest *SAUR_e* transcript levels of all of the lines tested. In the absence of TIBA D4-2 showed a 6-fold increase in *SAUR_e* transcript levels compared to wild type.

These results indicate that P6 stimulates the expression of the auxin responsive gene *SAUR_e*. This expression appears to be P6 dose dependent, and in P6 transgenic plants but not wild type, levels of *SAUR_e* transcripts are further stimulated 2-3-fold by TIBA treatment.



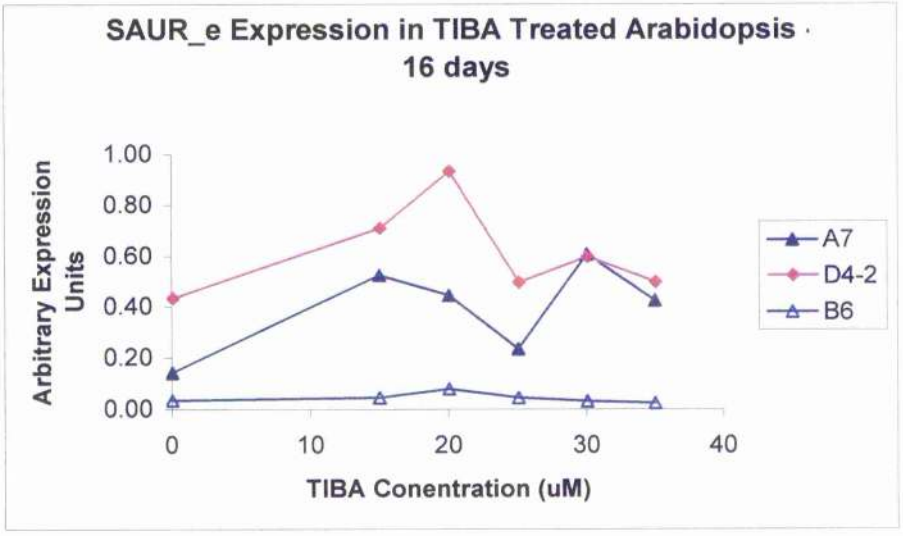
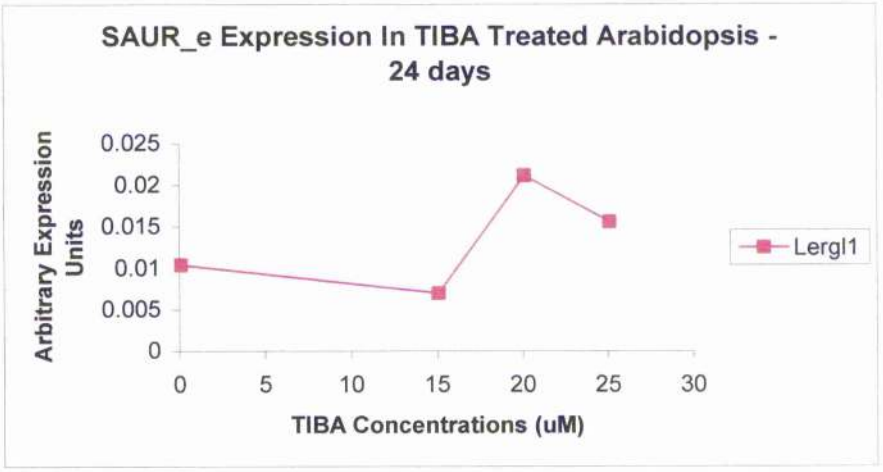


Fig 5.4 Transcript levels of the auxin responsive and TIBA responsive gene, *SAUR_e* in gene VI transgenics and wild types grown on TIBA concentrations ranging from 0 to 35μM. Levels are shown for 16 days after germination as quantified by RT-QPCR.



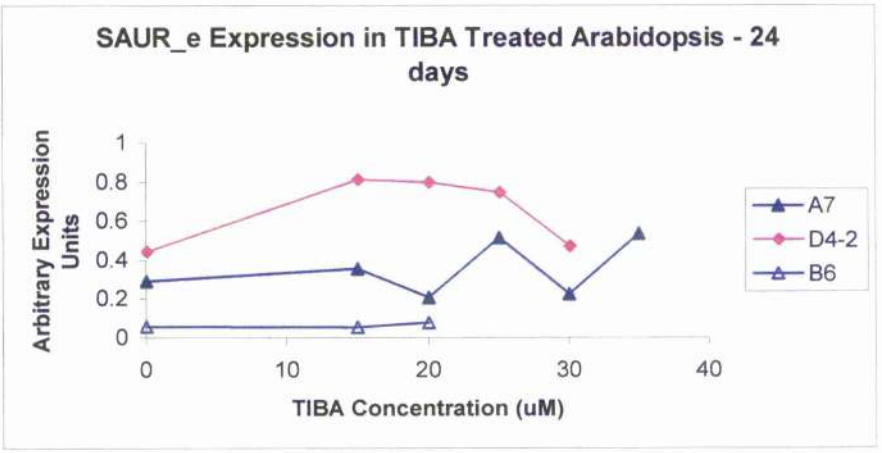


Fig 5.5 Transcript levels of the auxin responsive and TIBA responsive gene, *SAUR_e* in gene VI transgenics and wild types grown on TIBA concentrations ranging from 0 to 35μM. Levels are shown for 24 days after germination as quantified by RT-QPCR.

Genetic Analysis of the Interaction between P6 And Auxin (5.2.3)

To further characterize the interaction between P6 and auxin signalling and transport, and to try to identify at which point in the signalling pathway this interaction might occur, the P6 transgenic lines A7 and B6 were crossed with the auxin mutants, *aux1-100*, *aux1-7*, *tir1-1*, *tir2-2* and *tir3-1* (see Materials and Methods, 2.13). Controls were the P6 transgenics crossed with the wild types, Col-0 and WS. All of the auxin mutant alleles are recessive whilst 35S::gene VI is semi-dominant. Therefore, plants which are hemizygous for P6 are stunted and chlorotic but to a much lesser extent than those that have two copies of the transgene (Geri *et al.*, 2004). In crosses between P6 transgenics and wild type it was difficult to distinguish gene VI hemizygous plants from gene VI homozygous plants at the seedling stage; therefore the phenotyopes were scored as though the 35S::Gene VI transgene was a dominant allele. Thus the expected segregation ratio of chlorotic:green plants is 3:1 (see **Table 5.2**).

For each cross, the F2 progeny from independent F1 plants were grown on soil and assessed visually. Wild type Col-0 when crossed with A7 produced F2 offspring that segregated phenotypically close to the expected ratio 3:1. On average 71.9 (+/-1.9) percent of the plants showed the chlorotic phenotype normally associated with P6. This was also the case for the F2 generation of the *aux1-7* and *aux1-100* crosses (see **Table 5.2**) where 80.2± 4.2%) and 67.0 (+/-7.4) percent respectively showed a pale chlorotic phenotype. Neither *tir1-1* nor *tir2-2* produced successful F2 lines for analysis. However, *tir3-1* when crossed

with A7 gave F2 progeny with an aberrant segregation ratio. Instead of the expected 25 percent (1:3) showing a dark green phenotype 42.4 (+/- 0.7) percent of the plants showed a dark green phenotype. ANOVA indicates that this differs significantly from the expected 25 percent ($p<0.05$). Thus, the *tir3-1* mutation appears to be suppressing the P6 related chlorosis phenotype.

Cross	Number of Plants	Segregation Ration (expected ratio 3:1)		Average (+/- Std deviation)	
		Pale Green	Dark green	Pale Green	Dark green
Col-0	102	70.6	29.4		
	56	73.2	26.8	71.9 (1.9)	24.1(1.9)
aux1-7	54	81.5	18.5		
	86	75.6	24.4	80.2 (4.2)	18.8 (4.2)
	61	83.6	16.4		
aux1-100	54	59.3	40.7		
	46	63.0	37.0	67.0 (7.4)	33.0 (7.4)
	46	69.6	30.4		
	46	76.0	24.0		
tir3-1	98	58.2	41.8		
	77	57.1	42.9	42.3 (0.7)	57.7 (0.7)

Table 5.2 Segregation ratios of the F2 generation of P6/auxin crosses. Plants scored as either showing wild type phenotype (dark green) or P6 phenotype (pale green). Each line represents the phenotypic segregation for the progeny of individual F1 plants. All crosses were carried out using A7 as the P6 transgenic parent.

Summary (5.3)

CaMV symptom determinant P6 has been shown to interact with both ethylene and SA signalling pathways in Arabidopsis (Geri *et al.*, 2004; Love *et al.*, in preparation). The results reported in this chapter demonstrate that P6 also shows an interaction with auxin signalling.

When the P6 transgenic lines were grown on plates containing different concentrations of the auxin efflux inhibitor TIBA, transgenic plants exhibited a strong TIBA resistant phenotype. Resistance to TIBA appeared to be P6 dose-dependent. D4-2, which contains the highest levels of P6 protein, was the most resistant to TIBA and B6 which contains the lowest levels of P6 was the least resistant. These results also indicate that this resistance to TIBA is independent of the stunting and chlorosis phenotype, as D4-2, which expresses P6 from a mild strain of CaMV and is wild type in appearance, was highly resistant to TIBA. Interestingly B2-3 and B4-2, which contain the A7 transgene together with two alleles of an unlinked mutation (*cse*) that suppresses P6 dependent chlorosis and stunting, reverted almost to wild type sensitivity to TIBA. Therefore in addition to suppressing the chlorotic dwarf phenotype the mutations at the *CSE* locus results in a suppression of P6-dependent TIBA resistance. In contrast the *cse* allele does not suppress either ethylene or SA signalling interactions (Geri *et al.*, 2004; Love *et al.*, in preparation). It may be significant that *cse* mutants show a leaf curling phenotype (Geri *et al.*, 2004) which is reminiscent of several auxin signalling, and transport mutations e.g. *mdr1*, and the *phot1phot2* double mutants

(Noh *et al.*, 2001; Sakamoto and Briggs, 2002). This provides further evidence for a role for CSE in auxin signalling.

Expression of P6 also radically altered the transcript levels of an auxin responsive gene *SAUR_e*, whose expression is up-regulated in response to CaMV infection (See Chapter 4). Transcript levels of the *SAUR_e* gene were highly elevated (up to 30-fold) in the P6 lines compared to the wild types. Levels of expression were P6 dose dependent. D4-2 showed the highest levels of both P6 protein and *SAUR_e* expression and B6 showed the lowest. Interestingly, in contrast to the study reported in Genevestigator (Zimmermann *et al.*, 2004; Riken Group, 2004, unpublished; genevestigator chip # 113), *SAUR_e* transcript levels in wild type plants were low and showed no significant reduction in response to TIBA treatment. However, exposure of all three P6 transgenic lines to TIBA resulted in a further 2-3-fold increase in *SAUR_e* transcript levels. Therefore P6 was able to stimulate *SAUR_e* expression in the presence of TIBA.

Crosses between P6 transgenic lines A7 and B6 and the auxin signalling mutants provide further, albeit preliminary evidence for an interaction between P6 and auxin signalling. In one cross, *tir3-1* x A7 the F2 progeny segregated with an aberrant ratio. The increased proportion of green progeny in the F2 is consistent with a suppression of the chlorotic dwarf P6 phenotype in *tir3-1* homozygotes. *TIR3* maps to 2 cM on chromosome 3, and the gene VI transgene in A7 has been

roughly mapped to approximately 40 cM on the same chromosome (Laird and Milner, unpublished). Although both loci are located on chromosome 3 they are sufficiently far apart to behave as unlinked. If the *tir3-1* allele is indeed suppressing the P6-induced chlorosis and stunting phenotype, the predicted ratio of green: chlorotic seedlings should be 44:56 for two unlinked loci. This is very close to the observed ratio of 42:58. Therefore, although this evidence is preliminary, the segregation patterns of the F2 plants are consistent with an interaction between P6 and the product of the *TIR3* locus.

Therefore the results discussed above point to a specific role for *TIR3* in CaMV infection and suggest that it is somehow necessary for the development of systemic symptoms. An interaction between *TIR3* and the viral protein P6 which is known to be involved in symptom production is required. As no other components of the auxin transport and signalling pathway appeared to be involved it is likely that the auxin responses observed are as a consequence of this specific interaction. A possible mechanism is detailed in **Figure 5.6**.

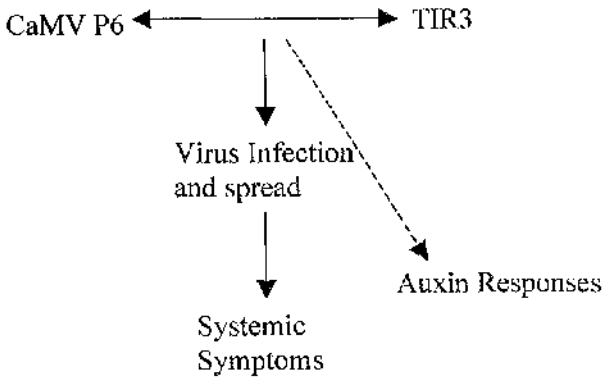


Fig 5.6 Possible mechanism showing the interaction of *TIR3* and CaMV P6 and the subsequent auxin responses that result from this interaction and the establishment of successful infection and systemic symptoms.

Chapter Six

General Discussion (6.1)

Auxin's Role in the Development of Compatible Virus Infection (6.1)

One of the main objectives of the work reported in this thesis was to assess the role of auxin in the host-virus interaction during compatible infections. To achieve this, a genetic approach was adopted and studies were carried out on a number of auxin signalling and transport mutant Arabidopsis lines. The initial aim was to assess the symptom response of both the wild-types and mutant lines when infected with CaMV and also to examine virus accumulation and spread within these plants. The second aim was to establish whether CaMV infection affected auxin signalling, transport or concentration and finally to determine mechanisms by which these responses occurred.

Historically, studying the role of auxin during virus infection involved the application of exogenous auxin compounds, or assays of auxin levels in infected plants (reviewed by Pennazio and Roggero, 1996). Many reports indicated that auxin treatment attenuated symptoms and also inhibited virus replication (reviewed by Fraser and Whenham, 1982; Nichols, 1952; Kutsky and Rawlins, 1950). However, it is worth noting that these responses were often achieved through the application of cytotoxic quantities of auxin analogues. For example, van Loon *et al.*, (1990) demonstrated that IAA and NAA when applied exogenously slightly reduced whereas 2,4-D strongly increased TMV replication in tobacco plants. The majority of investigations measured virus multiplication by infectivity bioassay, assessing the number of local lesions produced on a suitably reacting second host. This is not accurate enough to be

able to discriminate between small differences in virus concentration (Fraser and Whenham, 1982). Also the treatment of plants with growth regulators may have an indirect influence on the specific infectivity of the virus. For these reasons an accurate direct measurement of virus replication and accumulation is required.

One effective way of dissecting the role of auxin in this process is to use a genetic approach. A large number of mutant alleles, carrying defects in various aspects of auxin signalling and transport, are available. These mutants provide an invaluable tool for investigating the role of auxin in many different processes within the plant. They can provide a link to the causes of the alterations in host response observed during infection.

If auxin is responsible for symptoms or other responses to infection we might expect that disrupting auxin signalling and transport would lead to alterations in responses to infection. However experiments described in Chapter 3, taken together with additional unpublished work in the Milner lab, indicate that such mutants in general, do not differ from wild-type in either symptom phenotype or virus accumulation. The auxin mutants *axr4* and *axr2* which have been examined previously (Cecchini and Milner, unpublished) do not show any obvious differences in response to virus infection compared to wild-type. This was also found to be the case for the three *aux1* alleles and the transport inhibitor response mutants *tir1-1* and *tir2-2* examined here.These results suggest that most mutations that affect polar auxin transport or signalling do not affect host-virus

interactions. Therefore, auxin signalling may not play a major role in the susceptibility of plants to infection. However, *tir1-1* was found to have a p-value of 0.0703 which although greater than 0.05 and therefore usully regarded as not significant still indicates that there is a 93% chance that the result is significantly different from the wild-type and could implicate *TIR1* in the infection process.

One exception to this was *tir3-1* mutant plants. Here, symptom development was very significantly delayed and virus titres were much lower (up to 3-fold) compared to wild-type. Visualization of the virus by CaMV::GFP fluorescence confirmed that in *tir3-1*, cell to cell spread of CaMV was greatly reduced and delayed and movement of the virus into the vascular system was also delayed. As only *tir3-1*, of all the mutants tested, showed an altered response to CaMV this suggests that *TIR3* may play a more specific role in the host-virus interaction. *tir3-1* was originally identified in a screen for plants with altered responses to auxin transport inhibitors. *TIR3* is essential for either the synthesis, localization, or the function of the NPA binding site (Gil *et al.*, 2001). Recent research has shown (Gil *et al.*, 2001) that *tir3-1* is allelic to the *doc1-1* mutation. The *DOC1* (Dark Overexpreesing *CAB* 1) gene is a putative component of the light signal transduction pathways (Li *et al.*, 1994). Plants carrying mutations in this locus show overexpression of *CAB* genes in the dark (Li *et al.*, 1994). The locus has been recently renamed as *BIG* (Gil *et al.*, 2001) and is predicted to encode a large protein (560 KD) with significant similarity to the *Drosophila* protein Calossin/Pushover. Calossin is believed to be involved in the control of

synaptic transmission at the neuromuscular junction, in a process that requires the synaptic vesicle cycle (Xu *et al.*, 1998). Like synaptic transmission, the asymmetric distribution of auxin efflux carriers at the plasma membrane depends on targeted vesicle transport (Steinmann *et al.*, 1998) and recent work has shown that *BIG* is essential for proper positioning of the auxin efflux carrier, *PIN1*, at the plasma membrane (Paciorek *et al.*, 2005). *tir3* mutants also show defects in ethylene signalling and Kanyuka *et al.*, (2003) has suggested that rather than playing a *specific* role in auxin signalling, BIG may be involved in the control of vesicle transport and fusion, affecting a variety of signalling pathways. The results here all suggest that the *TIR3* locus is important for a successful CaMV infection to develop. Any interaction between auxin signalling and virus infection thus appears to be highly specific, and may not involve global changes in auxin levels and/or transport within the plant.

Fraser *et al.*, (1986) found a correlation between the level of symptoms and the level of virus accumulation in tobacco infected with TMV, although other studies have found that symptom severity may not be directly correlated to the level of virus accumulation within infected plants (Cecchini *et al.*, 1998; Fraser *et al.*, 1986). This suggests that several factors may influence symptom development and that these may vary, depending on the host and virus in question. Therefore, although a correlation between symptom response and virus titre was observed in the *tir3-1* mutant plants, the role of auxins might be more complex than simply altering symptom response through alterations in virus levels.

Auxin Responses are Altered by Compatible Virus Infection (6.1.2)

Auxin Responsive Gene Expression (6.1.2.1)

Virus infection not only elicits a symptom response within the host plant but can also lead to the altered expression of a number of host genes (Whitham *et al.*,2003; CaMV infection, Geri *et al.*, 1999). These include defence-related genes but also a range of genes which function in the growth and development of the host. A preliminary microarray investigation using Arabidopsis full genome chips identified several auxin responsive genes whose expression increased more than 3-fold in infected Arabidopsis.

The results presented in chapter 4 confirm that for three selected genes at least, CaMV infection of wild-type Arabidopsis (Col-0 and WS) stimulates increased transcript levels concomitant with virus accumulation within the plants. All of the genotypes examined showed this virus induced up-regulation in gene expression. However in the *aux1-100* mutant alleles and for the *tir3-1* plants this up-regulation was reduced although not abolished. *aux1-100* demonstrated a significant decrease in this virus-induced up-regulation for all three of the genes examined whereas *tir3-1*, showed a significant reduction in virus induced up-regulation for the *SAUR_e* gene only.

The developmental expression of *LAX2*, an auxin-responsive *AUX1* homologue (Bennett, unpublished), was also examined. In reporter plants where GUS expression was driven by a *LAX2* promoter, virus infection reduced the developmentally regulated expression of the *LAX2* gene. One interpretation of

these results is that auxin levels or activity are reduced in response to infection. However *LAX2* expression is regulated developmentally as well as in response to auxin levels, so the results observed might be due to a combination of factors rather than to direct changes in auxin levels or distribution within the plants.

These results point to an involvement of auxin transport or signalling in the host response to infection. However, whether this is as a direct consequence of alterations in auxin levels is not clear, and as discussed below, direct measurement of auxin levels in infected plants did not reveal any obvious virus-dependent changes.

Effect of CaMV infection on free IAA levels (6.1.2.2)

Previous attempts to ascertain the effect of virus infection on auxin levels have relied on techniques which were only semi-quantitative and therefore results were often inconsistant and contradictory. For example, Smith *et al.*, (1968) found reduced auxin activity in bean, sugarbeet and tomato plants when infected with Beet curly top geminivirus (BCTV). A bioassay was used and sap from infected plants was examined and its ability to cause phototropic bending, an auxin regulated process was assessed. However, substantial increases in auxin activity have sometimes been reported in infected plants showing severe symptoms (Jameson *et al.*, 2002). In both TSW- and TMV- infected tomato plants, Jones (1956) found auxin activities were higher than in healthy control plants, although the former were visibly stunted (Fraser and Whenham, 1982).

One difficulty in interpreting the results of these studies is in differentiating between primary and secondary changes in hormone concentrations, in particular because of the extreme severity of symptoms caused by many viruses (Jameson and Clarke, 2002). Also, the symptoms and host responses associated with disease differ greatly depending on the virus, host plant and environmental conditions. Therefore the contradictory results gathered previously may well be due to a combination of different experimental systems and differences in the techniques employed.

In the work described in chapter 4, levels of free IAA in infected and uninfected plants were assayed directly using GC-MS. Auxin levels and distribution were also assayed indirectly using DR5::GUS reporter lines. Neither of these approaches revealed any significant changes in auxin levels as a result of virus infection, and the DR5::GUS reporter lines failed to show any changes in auxin distribution. It is possible that virus infection may induce subtle changes in IAA levels or distribution within infected plants that were not picked up by this study. However a second possibility is that the changes in expression of auxin responsive genes in infected plants do not occur as a consequence of alterations in auxin levels or distribution, but are indicative of disruption of the signalling pathways *downstream* of auxin.

P6 Interacts With Auxin Signalling and Transport Pathways (6.1.3)

If virus infection is interacting directly with downstream components of the auxin signal transduction pathways, then how might this be achieved? One

potential interactor encoded by the CaMV genome is P6, which has been shown to be a symptom determinant as well as a host range and pathogenicity determinant (Rothnie *et al.*, 1994; Bonneville *et al.*, 1989; Scholthof *et al.*, 1992). The direct involvement of P6 in symptom development has been demonstrated by the expression of P6 from a transgene, which in the absence of infection leads to the development of symptom-like leaf mosaics, chlorosis and mottling (Baughman *et al.*, 1988; Goldberg *et al.*, 1991; Cecchini *et al.*, 1997). P6 has previously been shown to alter responses to the plant hormones ethylene (Geri *et al.*, 2004) and SA. The work reported in this thesis demonstrates that P6 also affects auxin-signalling. P6 transgenics are able to tolerate high concentrations of the auxin efflux inhibitor TIBA and compared to wild-type non-transgenic plants contain greatly elevated levels of transcripts for the auxin responsive gene, *SAUR_e*. The function of SAUR_eis still unknown but it encodes a calmodulin binding protein and it has similarities to ARG7 an auxin induced gene.

Interestingly, in the presence of the P6 suppressor mutation, *cse* plants containing the P6 transgene from line A7, reverted to wild-type sensitivity to TIBA. In contrast, the presence of the *cse* allele does not suppress the P6-dependent ethylene and SA-insensitivity phenotypes (Geri *et al.*, 2004, Love *et al.*, in preparation). In the absence of the P6 transgene, plants homozygous for the *cse-1* and *cse-2* mutant alleles show a leaf distortion phenotype (Geri *et al* 2004) reminiscent of two other mutants with lesions in auxin efflux, *mdr1* and *phot1 phot2* (Noh *et al.*, 2001; Sakamoto and Briggs, 2002), a weak ethylene-

insensitive phenotype (Geri *et al.*,2004), but apparently wild-type responses to SA (Love *et al.*, in preparation). These observations suggest that the interactions between P6, its suppressor locus, and components of hormone response signalling pathways are complex and not necessarily equivalent.

The preliminary genetic analysis, carried out to identify the components of the auxin signalling pathway involved in the interaction with P6, found that *tir3-1* plants when crossed with A7 transgenic Arabidopsis produced F2 progeny with an aberrant segregation ratio. It would appear from these results that the *tir3-1* mutation may, like *cse*, be able to suppress the P6-associated chlorosis and stunting phenotype. *TIR3* is required for correct localization of the auxin efflux carrier *PIN1* and may also play a more general role in vesicle trafficking. The possibility that P6 might interact directly with *TIR3*, affecting trafficking and other auxin related responses is intriguing although at this stage still entirely speculative.

Conclusions (6.2)

These results highlight the inconsistency faced by researchers investigating the auxin response and help to explain why the work to date has been inconclusive as to the role of auxin in the infection process. CaMV-infected Arabidopsis plants show some of the responses normally attributable to changes in auxin levels or distribution; however it appears that these responses may reflect interactions between virus components and components of auxin-signalling pathways as opposed to alterations in auxin levels *per se*.

The *TIR3* locus appears to be important for virus infection. Indeed, the effects observed in response to CaMV infection might even be a consequence of a direct interaction between *TIR3* and CaMV P6. There is good evidence that auxin interacts or cross-talks with many of the other plant hormones (Swarup et al., 2001). This cross talk allows the plant to fine-tune its signalling so that a variety of specific responses can occur to a wide range of factors. It is possible that the role of *TIR3* in vesicle trafficking might not only affect auxin responses but also other hormone related responses triggered during infection. A role for ethylene during CaMV infection of Arabidopsis has already been identified (Geri *et al.*, 2004; Love *et al* 2005) and so an involvement for auxin indirectly in these responses cannot be ruled out.

Future Work (6.3)

In order to explain fully how auxin signalling interacts with CAMV during infection a number of additional studies are required. Firstly to establish whether the auxin responses that occur are due to alterations in auxin levels or distribution, auxin levels need to be examined further. Studies looking at differences in auxin levels between tissue types and even different cells along with more sensitive techniques which could pick up even subtle changes in auxin levels would be needed. The work carried out so far suggests thet *TIR3* is important for infection with CaMV but it is still in doubt whether this is due to its role in auxin efflux as part of the carrier complex or due to its possible role in

vesicle trafficking. It would therefore be interesting to examine the responses of mutants with defects in the other components of the efflux carrier, for example with defects is *PIN*. The interaction between P6 and auxin also needs to be looked at in greater detail. The genetic analysis described was only preliminary and so would need to be repeated to confirm the interaction with *TIR3* and to help to clarify whether P6 directly interacts with the *TIR3* component of the auxin signalling pathway and that the alterations in auxin response are as a consequence of this. If P6 requires *TIR3* for trafficking it is possible that this component is also involved in the ethylene and SA response observed during infection. Investigation into the the role of *TIR3* in infection independent from the auxin response could offer insight in to the infection process.

References

References

Agama K, Beach S, Schoelz J, Leisner SM (2002) The 5 ' third of Cauliflower mosaic virus gene VI conditions resistance breakage in Arabidopsis ecotype tsu-0. Phytopathology 92: 190-196

Ahlquist P (2002) RNA-dependent RNA polymerases, viruses, and RNA silencing. Science 296: 1270-1273

Al Kaff NS, Covey SN (1994) Variation in biological properties of cauliflower mosaic-virus clones. J Gen Virol 75: 3137-3145

Al Kaff NS, Covey SN (1995) Biological diversity of cauliflower mosaic virus isolates expressed in two Brassica species. Plant Pathology 44: 516-526

Al Kaff NS, Turner DS, Covey SN (1997) Multiple pathogenic factors influence aphid transmission of cauliflower mosaic virus from infected plants. European Journal of Plant Pathology 103: 65-71

Al Kaff NS, Covey SN, Kreike MM, Page AM, Pinder R, Dale PJ (1998) Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. Science 279: 2113-2115

Alvarez ME, Pennell RI, Meijer PJ, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92: 773-784

Araki T (2001) Transition from vegetative to reproductive phase. Current Opinion in Plant Biology 4: 63-68

Armour SL, Melcher U, Pirone TP, Lyttle DJ, Essenberg RC (1983) Helper component for aphid transmission encoded by region-II of Cauliflower mosaic-virus DNA. Virology 129: 25-30

Ashby J, Boutant E, Seemanpillai M, Sambade A, Ritzenthaler C, Heinlein M (2006) Tobacco mosaic virus movement protein functions as a structural microtubule-associated protein. J Virol 80: 8329-8344

Baldacci E (1953) Le virosi delle piante come malattie dello sviluppo e della moltiplicazione vegetative: la degenerazione della patata. Nuovo giornale Botanico Italiano 60: 771-784

Baughman GA, Jacobs JD, Howell SH (1988) Cauliflower mosaic-virus gene-vi produces a symptomatic phenotype in transgenic tobacco plants. Proc Natl Acad Sci U S A 85: 733-737

Baulcombe D (2001) RNA silencing - Diced defence. Nature 409: 295-296

Baulcombe D (2002) Viral suppression of systemic silencing. Trends in Microbiology 10: 306-308

Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schulz B, Feldmann KA (1996) Arabidopsis AUX1 gene: A permease-like regulator of root gravitropism. Science 273: 948-950

Bennett MJ, Marchant A, May ST, Swarup R (1998) Going the distance with auxin: unravelling the molecular basis of auxin transport. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 353: 1511-1515

BenTal Y, Marco S (1980) Qualitative changes in cucumber gibberellins following Cucumber mosaic-virus infection. Physiological Plant Pathology 16: 327-336

Berger S, Bell E, Mullet JE (1996) Two methyl jasmonate-insensitive mutants show altered expression of AtVsp in response to methyl jasmonate and wounding. Plant Physiology 111: 525-531

Beuve M, Naibo B, Foulgocq L, Lapierre H (1999) Irrigated hybrid maize crop yield losses due to barley yellow dwarf virus-PAV luteovirus. Crop Science 39: 1830-1834

Bhalerao RP, Eklof J, Ljung K, Marchant A, Bennett M, Sandberg G (2002) Shoot-derived auxin is essential for early lateral root emergence in Arabidopsis seedlings. Plant Journal 29: 325-332

Blakeslee JJ, Peer WA, Murphy AS (2005) Auxin Transport. Current opinion in Plant Biology 8: 1-7

Blanc S, Cerutti M, Usmany M, Vlak JM, Hull R (1993a) Biological-activity of Cauliflower mosaic-virus aphid transmission factor expressed in a heterologous system. Virology 192: 643-650

Blanc S, Cerutti M, Chaahibi H, Louis C, DevauchelleG, Hull R (1993b) Gene-II product of an aphid-nontransmissible isolate of Cauliflower mosaic-virus expressed in a baculovirus system possesses aphid transmission factor activity. Virology 192: 651-654

Blanc S, Schmidt I, Kuhl G, Esperandieu P, Lebeurier G, Hull R, Cerutti M, Louis C (1993c) Paracrystalline structure of Cauliflower mosaic-virus aphid transmission factor produced both in plants and in a heterologous system and relationship with a solubilized active form. Virology 197: 283-292

Blee E (2002) Impact of phyto-oxylipins in plant defense. Trends in Plant Science 7: 315-321

Bleecker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in Arabidopsis-thaliana. Science 241: 1086-1089

Boevink P, Oparka KJ (2005) Virus-host interactions during movement processes. Plant Physiol 138: 1815-1821

Bonneville JM, Sanfacon H, Futterer J, Hohn T (1989) Posttranscriptional trans-activation in cauliflower mosaic virus. Cell 59: 1135-1143

Booker J, Chatfield S, Leyser O (2003) Auxin acts in xylem-associated or medullary cells to mediate apical dominance. Plant Cell 15: 495-507

Boyko V, Ashby JA, Suslova E, Ferralli J, Sterthaus O, Deom CM, Heinlein M (2002) Intramolecular complementing mutations in Tobacco mosaic virus movement protein confirm a role for microtubule association in viral RNA transport. J Virol 76: 3974-3980

Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC (1998) Viral pathogenicity determinants are suppressors of transgene silencing in nicotiana benthamiana. The EMBP Journal 17: 6739-6746

Broglio EP (1995) Mutational analysis of cauliflower mosaic virus gene VI - changes in host-range, symptoms, and discovery of transactivation-positive, noninfectious mutants. Mol Plant-Microbe Interact 8: 755-760

Bureau M, Leh V, Haas M, Geldreich A, Ryabova L, Yot P, Keller M (2004) P6 protein of Cauliflower mosaic virus, a translation reinitiator, interacts with ribosomal protein L13 from *Arabidopsis thaliana*. J Gen Virol 85: 3765-3775

Callaway A, Liu WN, Andrianov V, Stenzler L, Zhao JM, Wettlaufer S, Jayakumar P, Howell SH (1996) Characterization of cauliflower mosaic virus (CaMV) resistance in virus-resistant ecotypes of Arabidopsis. Mol Plant-Microbe Interact 9: 810-818

Callaway AS, Huang Z, Howell SH (2000) Host suppressors in *Arabidopsis thaliana* of mutations in the movement protein gene of Cauliflower mosaic virus. Mol Plant-Microbe Interact 13: 512-519

Carrington JC, Kasschau KD, Mahajan SK, Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell 8: 1669-1681

Cecchini E, Gong ZH, Geri C, Covey SN, Milner JJ (1997) Transgenic Arabidopsis lines expressing gene VI from cauliflower mosaic virus variants exhibit a range of symptom-like phenotypes and accumulate inclusion bodies. Mol Plant-Microbe Interact 10: 1094-1101

Cecchini E, Al Kaff NS, Bannister A, Giannakou ME, McCallum DG, Maule AJ, Milner JJ, Covey SN (1998) Pathogenic interactions between variants of cauliflower mosaic virus and *Arabidopsis thaliana*. J Exp Bot 49: 731-737

Cecchini E, Geri C, Love AJ, Coupland G, Covey SN, Milner JJ (2002) Mutations that delay flowering in Arabidopsis de-couple symptom response from cauliflower mosaic virus accumulation during infection. Mol Plant Pathol 3: 81-90

Champagne J, Benhamou N, Leclerc D (2004) Localization of the N-terminal domain of cauliflower mosaic virus coat protein precursor. Virology 324: 257-262

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene ETR1 - similarity of product to 2-component regulators. Science 262: 539-544

Chao QM, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell 89: 1133-1144

Chapdelaine Y, Hohn T (1998) The cauliflower mosaic virus capsid protein: Assembly and nucleic acid binding in vitro. Virus Genes 17: 139-150

Chapdelaine Y, Kirk D, Karsies A, Hohn T, Leclerc D (2002) Mutation of capsid protein phosphorylation sites abolishes cauliflower mosaic virus infectivity. J Virol 76: 11748-11752

Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC (2004) Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes & Development 18: 1179-1186

Chaudhry Z., Yoshioka T, Satoh S, Hase S, Ehara Y (1998) Stimulated ethylene production in tobacco (Nicotiana tabacum L. cv. Ky 57) leaves systemically infected with cucumber mosaic virus yellow strain. Plant Science 131: 123-130

Chellappan P, Vanitharani R, Fauquet CM (2004) Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. J Virol 78: 7465-7477

Cheng RH, Olson NH, Baker TS (1992) Cauliflower mosaic-virus - A-420 subunit(T = 7), multilayer structure. Virology 186: 655-668

Chivasa S, Carr JP (1998) Cyanide restores N gene-mediated resistance to tobacco mosaic virus in transgenic tobacco expressing salicylic acid hydroxylase. Plant Cell 10: 1489-1498

Citovsky V, Knorr D, Schuster G, ZambryskiP (1990) The p-30 movement protein of Tobacco mosaic-virus is a single-strand nucleic-acid binding-protein. Cell 60: 637-647

Citovsky V, Zambryski P (1991) How do plant-virus nucleic-acids move through intercellular connections. Bioessays 13: 373-379

Citovsky V (1999) Tobacco mosaic virus: a pioneer of cell-to-cell movement. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 354: 637-643

Collett CE, Harberd NP, Leyser O (2000) Hormonal interactions in the control of Arabidopsis hypocotyl elongation. Plant Physiol 124: 553-561

Covey SN, Lomonossoff GP, Hull R (1981) Characterization of Cauliflower mosaic-virus DNA-sequences which encode major polyadanylated transcripts. Nucleic Acids Res 9: 6735-6747

Covey SN, Hull R (1981) Transcription of cauliflower mosaic-virus DNA - detection of transcripts, properties, and location of the gene encoding the virus inclusion body protein. Virology 111: 463-474

Covey SN, Turner DS, Lucy AP, Saunders K (1990) Host regulation of the Cauliflower mosaic-virus multiplication cycle. Proc Natl Acad Sci U S A 87: 1633-1637

Covey SN, Al Kaff NS, Langara A, Turner DS (1997) Plants combat infection by gene silencing. Nature 385: 781-782

Covey SN (2000) Silencing genes silencing genes. Trends in Plant Science 5: 405-406

Covey SN, Al Kaff NS (2000) Plant DNA viruses and gene silencing. Plant Mol Biol 43: 307-322

Covey SN, McCallum DG, Turner DS, Al Kaff NS, Dale P, Cecchini E, Milner JJ (2000) Pararetrovirus-crucifer interactions: attack and defence or *modus vivendi*. Mol Plant Pathol 1: 77-86

Cronin S, Verchot J, Haldemancahill R, Schaad MC, Carrington JC (1995) Long-distance movement factor - a transport function of the potyvirus helper component proteinase. Plant Cell 7: 549-559

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature 435: 441-445

Dangl JL, Dietrich RA, Richberg MH (1996) Death don't have no mercy: Cell death programs in plant-microbe interactions. The Plant Cell 8: 1793-1807

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature 411: 826-833

Daubert S, Shepherd RJ, Gardner RC (1983) Insertional mutagenesis of the Cauliflower mosaic-virus genome. Gene 25: 201-208

Daubert S, Routh G (1990) Point mutations in Cauliflower mosaic-virus gene-VI confer host-specific symptom changes. Mol Plant-Microbe Interact 3: 341-345

Dautel S, Guidasci T, Pique M, Mougeot JL, Lebeurier G, Yot P, Mesnard JM (1994) The full-length product of Cauliflower mosaic-virus open reading frame-III is associated with the viral particle. Virology 202: 1043-1045

Davies PJ, Rubery PH (1978) Components of auxin transport in stem segments of *Pisum **sativum*** L. Planta 142: 211-219

De Wit PJGM (1997) Pathogen avirulence and plant resistance: a key role for recognition. Trends in Plant Science 2: 452-458.

del Pozo JC, Estelle M (1999) The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. Proc Natl Acad Sci U S A 96: 15342-15347

del Pozo JC, Estelle M (2000) F-box proteins and protein degradation: An emerging theme in cellular regulation. Plant Mol Biol 44: 123-128

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, and Ryals J (1994) A central role of salicylic acid in plant disease resistance. Science 266: 1247-1250

Delbarre A, Muller P, Imhoff V, GUERN J (1996) Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198: 532-541

Dempsey DA, Shah J, Klessig DF (1999) Salicylic acid and disease resistance in plants. Crit Rev Plant Sci 18: 547-575

Deom CM, Oliver MJ, Beachy RN (1987) The 30-kilodalton gene-product of Tobacco mosaic-virus potentiates virus movement. Science 237: 389-394

Detapia M, Himmelbach A, Hohn T (1993) Molecular dissection of the Cauliflower mosaic-virus translation transactivator. EMBO J 12: 3305-3314

Dharmasiri S, Swarup R, Mockaitis K, Dharmasiri N, Singh SK, Kowalchyk M, Marchant A, Mills S, Sandberg G, Bennett MJ, Estelle M (2006) AXR4 is required for localization of the auxin influx facilitator AUX1. Science 312: 1218-1220

Ding BA, LI QB, Nguyen L, Palukaitis P, Lucas WJ (1995) Cucumber mosaic-virus 3a protein potentiates cell-to-cell trafficking of CMVRNA in tobacco plants. Virology 207: 345-353

Ding B, Turgeon R, Parthasarathy MV (1992) Substructure of freeze-substituted plasmodesmata. Protoplasma 169: 28-41

Ding SW, Li WX, Symons RH (1995) A novel naturally-occurring hybrid gene encoded by a plant RNA virus facilitates long-distance virus movement. EMBO J 14: 5762-5772

Ding XS, Shintaku MH, Arnold SA, Nelson RS (1995) Accumulation of mild and severe strains of Tobacco mosaic-virus in minor veins of tobacco. Mol Plant-Microbe Interact 8: 32-40

Doares SH, Narvaezv Asquez J, Conconi A, Ryan CA (1995a) Salicylic-acid inhibits synthesis of proteinase-inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol 108: 1741-1746

Doares SH, Syrovets T, Weiler EW, Ryan CA (1995b) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc Natl Acad Sci U S A 92: 4095-4098

Dong XN (1998) SA, JA, ethylene, and disease resistance in plants. Current Opinion in Plant Biology 1: 316-323

Drucker M, Froissart R, Hebrard E, Uzest M, Ravallec M, Esperandieu P, Mani JC, Pugniere M, Roquet F, Fereres A, Blanc S (2002) Intracellular distribution of viral gene products regulates a complex mechanism of cauliflower mosaic virus acquisition by its aphid vector. Proc Natl Acad Sci U S A 99: 2422-2427

Dunez J, Sutic D (1988) Plum pox virus European Handbook of Plant Diseases, Blackwell, Oxford

Eckardt NA (2001) Auxin and the power of the proteasome in plants. Plant Cell 13: 2161-2163

Ecker JR (1995) The Ethylene signal-transduction pathway in plants. Science 268: 667-675

Eckert M, Kaldenhoff R (2000) Light-induced stomatal movement of selected *Arabidopsis thaliana* mutants. J Exp Bot 51: 1435-1442

Edlund A, Eklof S, Sundberg B, Moritz T, Sandberg G (1995) A microscale technique for gas-chromatography mass-spectrometry measurements of picogram amounts of indole-3-acetic-acid in plant-tissues. Plant Physiol 108: 1043-1047

Edwards MC, Fetch TG, Schwarz PB, Steffenson BJ (2001) Effect of Barley yellow dwarf virus infection on yield and malting quality of barley. Plant Disease 85: 202-207

Ephritikhine G, pagant S, Fujioka S, Takatsuto S, Lapous D, Caboche M, Kendrick RE,. Barbier-Brygoo H (1999) The sax1 mutation defines a new locus involved in the brassinosteroid biosynthesis pathway in Arabidopsis thaliana. The Plant journal 18: 315-320

Farmer EE, Ryan CA (1990) Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad. Sci USA 87: 7713-7716

Fenczik CA, Padgett HS, Holt CA, Casper SJ, Beachy RN (1995) Mutational analysis of the movement protein of Odontoglossum ringspot virus to identify a host-range determinant. Mol Plant-Microbe Interact 8: 666-673

Fraser RSS (1987) Biochemistry of virus-infected plants. RSP, Letchworth, UK

Fraser RSS, Whenham RJ (1982) Plant-growth regulators and virus-infection - a critical-review. Plant Growth Regulation 1: 37-59

Friml J, Benfey P, Benkova E, Bennett M, Berleth T, Geldner N, Grebe M, Heisler M, Hejatko J, Jurgens G, Laux T, Lindsey K, Lukowitz W, Luschnig C,

Offringa R, Scheres B, Swarup R, Torres-Ruiz R, Weijers D, Zazimalova E (2006) Apical-basal polarity: why plant cells don't stand on their heads. Trends in Plant Science 11: 12-14

Frommer WB, Hummel S, Reismeier JW (1993) Expression and cloning in yeast a cDNA encoding a broad specificity amino acid permease from Arabidopsis thaliana. Proc Natl Acad Sci USA 90: 5944-5948

Fujita H, Syono K (1996) Genetic analysis of the effects of polar auxin transport inhibitors on root growth in *Arabidopsis thaliana*. Plant and Cell Physiology 37: 1094-1101

Gadea J, Mayda ME, Conejero V,Vera P(1996) Characterization of defense-related genes ectopically expressed in viroid-infected tomato plants. Mol Plant-Microbe Interact. 9:409-415.

Gardner CO, Shockey MW, EssenderG RC, Melcher UK (1979) Characterization of Cauliflower mosaic virus isolate CM4-184. Plant Physiol 63: 118

Geisler M, Blakeslee JJ, Bouchard R, Lee OR, Vincenzetti V, Bandyopadhyay A, Titapiwatanakun B, Peer WA, Bailly A, Richards EL, Ejendal KFK, Smith AP, Baroux C, Grossnuklaus U, Muller A, Hrycyna CA, Dudler R, Murphy AS, Martinola E (2005) Cellular efflux of auxin catalyzed by the Arabidopsis MDr/PGP transporter AtPGP1. The Plant Journal 44: 179-194

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, and Jürgens G (2003). The *Arabidopsis* GNOM ARF-GEF Mediates Endosomal Recycling, Auxin Transport, and Auxin-Dependent Plant Growth. Cell **112**, pp. 219–230.

Gelvin SB (1998) The introduction and expression of transgenes in plants. Current Opinion in Biotechnology 9: 227-232

Genoud T, Metraux, JP (1999) Crosstalk in plant cell signalling: structure and function of the genetic network. Trends in Plant Science 4: 503–507.

Geraats BPJ, Bakker PAHM, van Loon LC (2002) Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*. Mol Plant-Microbe Interact 15: 1078-1085

Geri C, Cecchini E, Giannakou ME, Covey SN, Milner JJ (1999) Altered patterns of gene expression in Arabidopsis elicited by cauliflower mosaic virus

(CaMV) infection and by a CaMV gene VI transgene. Mol Plant-Microbe Interact 12: 377-384

Geri C, Love AJ, Cecchini E, Barrett SJ, Laird J, Covey SN, Milner JJ (2004) Arabidopsis mutants that suppress the phenotype induced by transgene-mediated expression of cauliflower mosaic virus (CaMV) gene VI are less susceptible to CaMV-infection and show reduced ethylene sensitivity. Plant Mol Biol 56: 111-124

Gibbs A (1976) Virus and plasmodesmata. In Intercellular Communication in Plants: Studies on Plasmodesmata (eds Gunning BES, Robards RW): 149-164. Springer-Verlag, Berlin/Heidelberg/New York

Gifford RM, Evans LT (1981) Photosynthesis, caron portioning, and yield. Annual Review of Plant Physiology 32: 485-509

Gil P, Dewey E, Friml J, Zhao Y, Snowden KC, Putterill J, Palme K, Estelle M, Chory J (2001) BIG: a calossin-like protein required for polar auxin transport in *Arabidopsis*. Genes and Development 15: 1985-1997

Gilbertson RL, Lucas WJ (1996) How do viruses traffic on the 'vascular highway'? Trends in Plant Science 1: 260-268

Glazebrook J, Weigel D (2002) Arabidopsis: A laboratory manual. In J Glazebrook, D Weigel, eds. Cold Spring Harbor Press, Cold Spring Harbor NY, pp 86

Goldberg KB, Kiernan J, Shepherd RJ (1991) A disease syndrome associated with expression of gene-VI of caulimoviruses may be a nonhost reaction. Mol Plant-Microbe Interact 4: 182-189

Grant M, Lamb C (2006) Systemic immunity. Current Opinion in Plant Biology 9: 414-420

Gray WM, Estelle M (2000) Function of the ubiquitin-proteasome pathway in auxin response. Trends Biochem Sci 25: 133-138

Gray WM, Kepinski S, Rouse D, Leyser O, Estelle M (2001) Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins. Nature 414: 271-276

Grieve (1936) Spotted wilt virus and the hormone heteroauxin. Nature 108: 129

Guerra-Peraza O, de Tapia M, Hohn T, Hemmings-Mieszczak M (2000) Interaction of the Cauliflower mosaic virus coat protein with the pregenomic RNA leader. J Virol 74: 2067-2072

Guidasci T, Mougeot JL, lebeurier G, Mesnard JM (1992) processing of the minor capsid protein of the cauliflower mosaic virus requires a cysteine proteinase. Research in Virology 143: 361-370

Guilfoyle TJ, HagenG, LI Y, Ulmasov T, Liu ZB, Strabala T, Gee M (1993) Auxin-regulated transcription. Australian Journal of Plant Physiology 20: 489-502

Gundlach H, Muller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant-cell cultures. Proc Natl Acad Sci U S A 89: 2389-2393

Gunning BES, Robards RW (1976) Intercellular communication in plants: Studies on plasmodesmata. Springer-Verlag, Berlin

Gupta V, Willits MG, Glazebrook J (2000) *Arabidopsis thaliana* EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: Evidence for inhibition of jasmonic acid signaling by SA. Mol Plant-Microbe Interact 13: 503-511

Haas M, Bureau M, Geldreich A, Yot P, Keller M (2002) Cauliflower mosaic virus: still in the news. Mol Plant Pathol 3: 419-429

Haas M, Geldreich A, Bureau M, Dupuis L, Leh W, Vetter G, Kobayashi K, Hohn T, Ryabova L, Yot P, Keller M (2005) The open reading frame VI product

of Cauliflower mosaic virus is a nucleocytoplasmic protein: Its N terminus mediates its nuclear export and formation of electron-dense viroplasms. Plant Cell 17: 927-943

Hadfi K, Speth V, Neuhaus G (1998) Auxin-induced developmental patterns in Brassica juncea embryos. Development 125: 879-887

Hagen G, Martin G, Li Y, Guilfoyle TJ (1991) Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. Plant Mol Biol 17: 567-579

Hammond-Kosack K, Jones JDG (2000) Responses to plant pathogens. In Biochemistry and Molecular Biology of Plants, B.B., Buchanan, W. Gruissem, and R.L. Jones, eds (Rockville, MD: American Society of Plant Physiologists), pp. 1102–1156.

Harper RM, Stowe-Evans EL, Luesse DR, Muto H, Tatematsu K, Watahiki MK, Yamamoto K, Liscum E (2000) The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial Arabidopsis tissue. Plant Cell 12: 757-770

Heath MC (2000) Hypersensitive response-related death. Plant Mol Biol 44: 321-334

Hebrard E, Drucker M, Leclerc D, Hohn T, Uzest M, Froissart R, Strub JM, Sanglier S, van Dorsselaer A, Padilla A, Labesse G, Blanc S (2001) Biochemical characterization of the helper component of Cauliflower mosaic virus. J Virol 75: 8538-8546

Hemmings-Mieszczak M, Steger G, Hohn T (1998) Regulation of CaMV 35 S RNA translation is mediated by a stable hairpin in the leader. RNA-A Publication of the RNA Society 4: 101-111

Hemmings-Mieszczak M, Steger G, Hohn T (1997) Alternative structures of the cauliflower mosaic virus 35 S RNA leader: Implications for viral expression and replication. Journal of Molecular Biology 267: 1075-1088

Hertel R (1995) Auxin-Binding Protein-1 is a red herring. J Exp Bot 46: 461-462

Himmelbach A, Chapdelaine Y, Hohn T (1996) Interaction between cauliflower mosaic virus inclusion body protein and capsid protein: Implications for viral assembly. Virology 217: 147-157

Hobbie LJ (1998) Auxin: Molecular genetic approaches in Arabidopsis. Plant Physiology and Biochemistry 36: 91-102

Hooft van Huijsduijnen RAM, Albias SW, De Rijk RH, Bol JF (1986) Induction by salicylic acid of pathogenesis-related proteins and resistance to alfalfa mosaic virus infection in various plant species. J Gen Virol 67: 2135-2143

Hohn T, Hohn B, Pfeiffer P (1985) Reverse transcription in CaMV. Trends Biochem Sci 10: 205-209

Hohn T, Futterer J (1997) The proteins and functions of plant pararetroviruses: Knowns and unknowns. Crit Rev Plant Sci 16: 133-161

Hohn T, Park HS, Guerra-Peraza O, Stavolone L, Pooggin MM, Kobayashi K, Ryabova LA (2001) Shunting and controlled reinitiation: The encounter of cauliflower mosaic virus with the translational machinery. Cold Spring Harbor Symposia on Quantitative Biology 66: 269-276

Hua J, Chang C, Sun Q, Meyerowitz EM (1995) Ethylene insensitivity conferred by Arabidopsis ERS gene. Science 269: 1712-1714

Hua J, Sakai H, Nourizadeh S, Chen QHG, Bleecker AB, Ecker JR, Meyerowitz EM (1998) EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell 10: 1321-1332

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94: 261-271

Huang Z, Andrianov VM, Han Y, Howell SH (2001) Identification of Arabidopsis proteins that interact with the cauliflower mosaic virus (CaMV) movement protein. Plant Mol Biol 47: 663-675

Hull R (1989) The movement of viruses in plants. Annual Review of Phytopathology 27: 213-240

Hull R (2002) Matthews Plant Virology, Fourth Edition. Academic Press, New York

Hull R, Covey SN (1983) Does Cauliflower mosaic-virus replicate by reverse transcription. Trends Biochem Sci 8: 119-121

Hull R, Covey SN (1985) Cauliflower mosaic-virus - pathways of infection. Biocssays 3: 160-163

Hull R, Covey SN, Maule AJ (1987) Structure and replication of Caulimovirus genomes. J Cell Sci 213-229

Hull R, Covey SN (1995) Retroelements: Propagation and adaptation. Virus Genes 11: 105-118

Ikeda T, Yakushiji H, Oda M, Taji A, Imada S (1999) Growth dependence of ovaries of facultatively parthenocarpic eggplant in vitro on indole-3-acetic acid content. Sci Hort 79: 143-150

Jacquot E, Geldreich A, Keller M, Yot P (1998) Mapping regions of the cauliflower mosaic virus ORF III product required for infectivity. Virology 242: 395-402

Jameson PE (2000) Cytokinins and auxins in plant-pathogen interactions - An overview. Plant Growth Regulation 32: 369-380

Jameson PE, Clarke SF (2002) Hormone-virus interactions in plants. Crit Rev Plant Sci 21: 205-228

Ji LH, Ding SW (2001) The suppressor of transgene RNA silencing encoded by Cucumber mosaic virus interferes with salicylic acid-mediated virus resistance. Mol Plant-Microbe Interact 14: 715-724

Johal GS, Hulbert SH, Briggs SP (1995) Disease lesion mimics of maize – A model for cell-death in plants. Bioessays 17: 685-692

Johnson and Ecker (1998) The ethylene gas signal transduction pathway: A molecular perspective, Annual Reviews in Genetics 32: 227-254.

Jones (1956) Studies on the auxin levels of healthy and virus infected plants, Dissertation Abstracts 16: 1567-1568

Jouanneau JP, Lapous D, Guern J (1991) In plant-protoplasts, the spontaneous expression of defense reactions and the responsiveness to exogenous elicitors are under auxin control. Plant Physiol 96: 459-466

Kachroo P, Yoshioka K, Shah J, Dooner HK, Klessig DF (2000) Resistance to turnip crinkle virus in Arabidopsis is regulated by two host genes and is salicylic acid dependent but NPR1, ethylene, and jasmonate independent. Plant Cell 12: 677-690

Karsies A, Hohn T, Leclerc D (2001) Degradation signals within both terminal domains of the cauliflower mosaic virus capsid protein precursor. Plant Journal 27: 335-343

Karsies A, Merkle T, Szurek B, Bonas U, Hohn T, Leclerc D (2002) Regulated nuclear targeting of cauliflower mosaic virus. J Gen Virol 83: 1783-1790

Kasteel DTJ, Perbal MC, Boyer JC, Wellink J, Goldbach RW, Maule AJ, vanLent JWM (1996) The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells. J Gen Virol 77: 2857-2864

Keller P, Luttge U (1991) Stress-physiology of sugar-beet plants (*Beta-vulgaris* L) in relation to rhizomania disease .1. General description and gas-exchange measurements. Angewandte Botanik 65: 59-73

Kepinski S, Leyser O (2002) Ubiquitination and auxin signaling: A degrading story. Plant Cell 14: S81-S95

Kepinski S, Leyser O (2003) SCF-mediated proteolysis and negative regulation in ethylene signaling. Cell 115: 647-648

Kepinski S, Leyser O (2003) Plant development - An axis of auxin. Nature 426: 132-+

Kepinski S, Leyser O (2004) Auxin-induced SCFTIR1-Aux/IAA interaction involves stable modification of the SCFTIR1 complex. Proc Natl Acad Sci U S A 101: 12381-12386

Kepinski S, Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435: 446-451

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in arabidopsis, encodes a member of the RAF family of protein-kinases. Cell 72: 427-441

Kiraly L, Cole AB, Bourque JE, Schoelz JE (1999) Systemic cell death is elicited by the interaction of a single gene in *Nicotiana clevelandii* and gene VI of Cauliflower mosaic virus. Mol Plant-Microbe Interact 12: 919-925

Kiraly L, Cole AB, Schoelz JE (2003) Temporal expression of PR-1 in *Nicotiana edwardsonii* - a dominant Mendelian trait correlated with enhanced resistance to virus-induced necrotization and increases in salicylic acid levels. Free Radical Research 37: 16

Kisslaszlo Z, Blanc S, Hohn T (1995) Splicing of Cauliflower mosaic virus 35S RNA is essential for viral infectivity. EMBO J 14: 3552-3562

Klein PG, Klein RR, Rodriguezcerezo E, Hunt AG, Shaw JG (1994) Mutational analysis of the Tobacco vein mottling virus genome. Virology 204: 759-769

Knoester M, van Loon LC, van den Heuvel J, Hennig J, Bol JF, Linthorst HJM (1998) Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proc Natl Acad Sci U S A 95: 1933-1937

Knoester M, Linthorst HJM, Bol JF, van Loon LC (2001) Involvement of ethylene in lesion development and systemic acquired resistance in tobacco during the hypersensitive reaction to tobacco mosaic virus. Physiological and Molecular Plant Pathology 59: 45-57

Kobayashi K, Tsuge S, Nakayashiki H, Mise K, Furusawa I (1998) Evidence for a dual strategy in the expression of cauliflower mosaic virus open reading frames I and IV. Microbiol Immunol 42: 329-334

Kobayashi K, Tsuge S, Stavolone L, Hohn T (2002) The cauliflower mosaic virus virion-associated protein is dispensable for viral replication in single cells. J Virol 76: 9457-9464

Kobayashi K, Hohn T (2003) Dissection of cauliflower mosaic virus transactivator/viroplasmin reveals distinct essential functions in basic virus replication. J Virol 77: 8577-8583

Kobayashi K, Hohn T (2004) The avirulence domain of cauliflower mosaic virus transactivator/viroplasmin is a determinant of viral virulence in susceptible hosts. Mol Plant-Microbe Interact 17: 475-483

Koch JR, Sherzer AJ, Eshita SM, Davis KR (1998) Ozone sensitivity in hybrid poplar is correlated with a lack of defense-gene activation. Plant Physiol 118: 1243–1252

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Current Opinion in Plant Biology 5: 325-331

Kuriger WE, Agrios GN (1977) Cytokinin levels and kinetin-virus interactions in Tobacco ringspot virus-infected cowpea plants. Phytopathology 67: 604-609

Lamb C, Dixon RA (1997) The oxidative burst in disease resistance. Annu Rev Plant Physiol 48: 251-275

Laval V, Koroleva OA, Murphy E, Lu C, Milner JJ, Hooks MA, Tomos DA (2002) Distribution of actin gene isoforms in the Arabidopsis leaf measured in microsamples from intact individual cells. Planta 215: 287-292

Lawton KA, Potter SL, Uknes S, Ryals J (1994) Acquired-resistance signal-transduction in Arabidopsis is ethylene independent. Plant Cell 6: 581-588

Leclerc D, Burri L, Kajava AV, Mougeot JL, Hess D, Lustig A, Kleemann G, Hohn T (1998) The open reading frame III product of cauliflower mosaic virus forms a tetramer through a N-terminal coiled-coil. Journal of Biological Chemistry 273: 29015-29021

Leclerc D, Chapdelaine Y, Hohn T (1999) Nuclear targeting of the cauliflower mosaic virus coat protein. J Virol 73: 553-560

Leclerc D, Stavolone L, Meier E, Guerra-Peraza O, Herzog E, Hohn T (2001) The product of ORF III in cauliflower mosaic virus interacts with the viral coat protein through its C-terminal proline rich domain. Virus Genes 22: 159-165

Lee JM, Hartman GL, Domier LL, Bent AF (1996) Identification and map location of TTR1, a single locus in Arabidopsis thaliana that confers tolerance to tobacco ringspot nepovirus. Molecular Plant Microbe Interactions 9: 729-735.

Leh V, Jacquot E, Geldreich A, Hermann T, Leclerc D, Cerutti M, Yot P, Keller M, Blanc S (1999) Aphid transmission of cauliflower mosaic virus requires the viral PIII protein. EMBO J 18: 7077-7085

Leh V, Yot P, Keller M (2000) The cauliflower mosaic virus translational transactivator interacts with the 60S ribosomal subunit protein L18 of *Arabidopsis thaliana*. Virology 266: 1-7

Leh V, Jacquot E, Geldreich A, Kaas M, Blanc S, Keller M, Yot P (2001) Interaction between the open reading frame III product and the coat protein is required for transmission of cauliflower mosaic virus by aphids. J Virol 75: 100-106

Lehman A, Black R, Ecker JR (1996) HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. Cell 85: 183-194

Leisner SM, Howell SH (1992) Symptom variation in different *Arabidopsis thaliana* ecotypes produced by Cauliflower mosaic-virus. Phytopathology 82: 1042-1046

Leisner SM, Turgeon R, Howell SH (1992) Long-distance movement of Cauliflower mosaic-virus in infected turnip plants. Mol Plant-Microbe Interact 5: 41-47

Leisner SM, Turgeon R (1993) Movement of virus and photoassimilate in the phloem - a comparative-analysis. Bioessays 15: 741-748

Leyser O, Lincoln C, TimpteC, Turner J, Lammer D, Estelle M (1993) Molecular characterization of the AXR1 gene of *Arabidopsis thaliana*. Journal of Cellular Biochemistry 41

Leyser O (1997) Auxin: Lessons from a mutant weed. Physiologia Plantarum 100: 407-414

Leyser O (1998) Auxin signalling: Protein stability as a versatile control target. Current Biology 8: R305-R307

Leyser O, Berleth T (1999) A molecular basis for auxin action. Seminars in Cell & Developmental Biology 10: 131-137

Leyser O (1999) Plant hormones: Ins and outs of auxin transport. Current Biology 9: R8

Leyser O, Deng XW (2000) Cell signalling and gene regulation - New directions in plant signalling - Editorial overview. Current Opinion in Plant Biology 3: 351-352

Leyser O (2001) Auxin signalling: the beginning, the middle and the end. Current Opinion in Plant Biology 4: 382-386

Leyser O (2002) Molecular genetics of auxin signaling. Annual Review of Plant Biology 53: 377-398

Leyser O (2003) Regulation of shoot branching by auxin. Trends in Plant Science 8: 541-545

Leyser O (2005)a The fall and rise of apical dominance - Commentary. Current Opinion in Genetics & Development 15: 468-471

Leyser O (2005b) Auxin distribution and plant pattern formation: How many angels can dance on the point of PIN? Cell 121: 819-822

Leyser O (2006) Dynamic integration of auxin transport and signalling. Current Biology 16: R424-R433

Li HM, Altschmeid L, Chory J (1994) Arabidopsis mutants define downstream branches in the phototransduction pathway. Genes and Development 8: 339-349

Li HW, Li WX, Ding SW (2002) Induction and suppression of RNA silencing by an animal virus. Science 296: 1319-1321

Li YC, Korol AB, Fahima T, Nevo E (2004) Microsatellites within genes: Structure, function, and evolution. Molecular Biology and Evolution 21: 991-1007

Li YZ, Leisner SM (2002) Multiple domains within the Cauliflower mosaic virus gene VI product interact with the full-length protein. Mol Plant-Microbe Interact 15: 1050-1057

Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R (2004) Jasmonate-insensitive1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16: 1938-1950

Lough TJ, Shash K, Xoconostle-Cazares B, Hofstra KR, Beck DL, Balmori E, Forster RLS, Lucas WJ (1998) Molecular dissection of the mechanism by which potexvirus triple gene block proteins mediate cell-to-cell transport of infectious RNA. Mol Plant-Microbe Interact 11: 801-814

Love AJ, Yun BW, Laval V, Loake GJ, Milner JJ (2005) Cauliflower mosaic virus, a compatible pathogen of Arabidopsis, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. Plant Physiol 139: 935-948

Lucas WJ, Gilbertson RL (1994) Plasmodesmata in relation to viral movement within leaf tissue. Annual Review of Phytopathology 32: 387-411

Lucas WJ (2006) Plant viral movement proteins: Agents for cell-to-cell trafficking of viral genomes. Virology 344: 169-184

Marchant A, Bennett MJ (1998) The Arabidopsis AUX1 gene: a model system to study mRNA processing in plants. Plant Mol Biol 36: 463-471

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ (1999) AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. EMBO J 18: 2066-2073

Marchant A, Bhalerao R, Casimiro I, Eklof J, Casero PJ, Bennett M, Sandberg G (2002) AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. Plant Cell 14: 589-597

Marco S, Levy D (1979) Involvement of ethylene in the development of Cucumber mosaic virus-induced chlorotic lesions in cucumber cotyledons. Physiological Plant Pathology 14: 235-244

Mattsson J, Ckurshumova W, Berleth T (2003) Auxin signaling in Arabidopsis leaf vascular development. Plant Physiology 131: 1327-1339

Maule AJ (1991) Virus movement in infected plants. Crit Rev Plant Sci 9: 457-473

Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc Natl Acad Sci 96: 8271-8276

Mayda E, Marques C, Conejero V, Vera P (2000) Expression of a pathogen-induced gene can be mimicked by auxin insensitivity. Mol Plant-Microbe Interact 13: 23-31

Melcher U (1989) Symptoms of Cauliflower mosaic-virus infection in *Arabidopsis thaliana* and turnip. Botanical Gazette 150: 139-147

Melcher U (2000) The '30K' superfamily of viral movement proteins. J Gen Virol 81: 257-266

Moissiard G, Voinnet O (2004) Viral suppression of RNA silencing in plants. Mol Plant Pathol 5: 71-82

Morozov SY, Solovyev AG (2003) Triple gene block: modular design of a multifunctional machine for plant virus movement. J Gen Virol 84: 1351-1366

Morris DA, Rubery PH, Jarman J, Sabater M (1991) Effects of inhibitors of protein-synthesis on transmembrane auxin transport in *Cucurbita-pepo L* hypocotyl segments. J Exp Bot 42: 773-783

Muday GK, DeLong A (2001) Polar auxin transport: controlling where and how much. Trends in Plant Science 6: 535-542

Murphy AM, Gilliland A, Eng Wong C, West J, Singh DP, Carr JP (2001) Signal transduction in resistance to plant viruses. European Journal of Plant Pathology 107: 121-128

Murphy AM, Carr JP (2002) Salicylic acid has cell-specific effects on Tobacco mosaic virus replication and cell-to-cell movement. Plant Physiol 128: 552-563

Napier RM (2005) TIRs of joy: new receptors for auxin. Bioassays 27: 1213-1217

Naylor M, Murphy AM, Berry JO, Carr JP (1998) Salicylic acid can induce resistance to plant virus movement. Mol Plant-Microbe Interact 11: 860-868

Nelson RS, Li GX, Hodgson RAJ, Beachy RN, Shintaku MH (1993) Impeded phloem-dependent accumulation of the masked strain of Tobacco mosaic-virus. Mol Plant-Microbe Interact 6: 45-54

Nelson RS, van Bel AJE (1998) The mystery of virus trafficking into, through and out of the vascular tissue. Prog Bot 59: 476-533

Németh M (1994) History and importance of plum pox virus in stone-fruit production. Bulletin OEPP/EPPO Bulletin 24: 525-536

Niki T, Mitsuhara I, Seo S, Ohtsubo N, Ohashi Y (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. Plant and Cell Physiology 39: 500-507

Nishiguchi M, Motoyoshi F, Oshima N (1978) Behavior of a temperature sensitive strain of Tobacco mosaic-virus in tomato leaves and protoplasts. J Gen Virol 39: 53-61

Noh B, Murphy AS, Spalding EP (2005) Multidrug Resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development. The Plant Cell 13: 2441-2454

Nurnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunological Reviews 198: 249-266

O'Donnell PJ, Schmelz EA, Moussatche P, Lund ST, Jones JB, Klee HJ (2003b) Susceptible to intolerance - a range of hormonal actions in a susceptible Arabidopsis pathogen response. Plant Journal 33: 245-257

Ohtsubo N, Mitsuhara I, Koga M, Seo S, Ohashi Y (1999) Ethylene promotes the necrotic lesion formation and basic PR gene expression in TMV-infected tobacco. Plant and Cell Physiology 40: 808-817

Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y (1991) Requirement of the auxin polar transport-system in early stages of *Arabidopsis* floral bud formation. Plant Cell 3: 677-684

Oparka KJ (2004) Getting the message across: how do plant cells exchange macromolecular complexes? Trends in Plant Science 9: 33-41

Oparka KJ, Roberts AG (2001) Plasmodesmata. A not so open-and-shut case. Plant Physiol 125: 123-126

Paciorek T, Zazímalová E, Ruthardt N, Petrásek J, Stierhof YD, Kleine-Vehn J, Morris DA, Emans N, Jürgens G, Geldner N (2005). Auxin inhibits endocytosis and promotes its own efflux from cells. Nature 435: 1251 -1256

Padmanabhan MS, Gorepoker SP, Golem S, Shiferaw H, Culver JN (2005) Interaction of the tobacco mosaic virus replicase protein with the Aux/IAA protein PAPI/IAA26 is associated with disease development. J Virol 79: 2549-2558

Padmanabhan MS, Shiferaw H, Culver JN (2006) The Tobacco mosaic virus replicase protein disrupts the localization and function of interacting Aux/IAA proteins. Mol Plant-Microbe Interact 19: 864-873

Palanichelvam K, Schoelz JE (2002) A comparative analysis of the avirulence and translational transactivator functions of gene VI of Cauliflower mosaic virus. Virology 293: 225-233

Park HS, Himmelbach A, Browning KS, Hohn T, Ryabova LA (2001) A plant viral "reinitiation" factor interacts with the host translational machinery. Cell 106: 723-733

Parry G, Marchant A, May S, Swarup R, Swarup K, James N, Graham N, Allen T, Martucci T, Yemm A, Napier R, Manning K, King G, Bennett M (2001a) Quick on the uptake: Characterization of a family of plant auxin influx carriers. Journal of Plant Growth Regulation 20: 217-225

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. Plant Cell 15: 939-951

Pennazio S, Roggero P (1996) Plant hormones and plant virus diseases. The auxins. Microbiologica 19: 369-378

Pennazio S, Roggero P (1998) Endogenous changes in citokinin activity in systemically virus-infected plants. Microbiologica 21: 419-426

Penninckx IAMA, Thomma BPHJ, Buchala A, Metraux JP, Broekaert WF (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell 10: 2103-2113

Perbal MC, Thomas CL, Maule AJ (1993) Cauliflower mosaic-virus gene-I product (P1) forms tubular structures which extend from the surface of infected protoplasts. Virology 195: 281-285

Petrásek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertová D, Wisniewska J, Tadele Z, Kubes M, Covanová M, Dhonukshe P, Skupa P, Benková E, Perry L, Krecek P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zazímalová E, Friml J (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312: 914-918

Petty ITD, Jackson AO (1990a) Mutational analysis of Barley stripe mosaic-virus RNA-beta. Virology 179: 712-718

Petty ITD, Jackson AO (1990b) 2 forms of the major Barley stripe mosaic-virus nonstructural protein are synthesized *in vivo* from alternative initiation codons. Virology 177: 829-832

Pickett FB, Wilson AK, Estelle M (1990) The AUX1 mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiol 94: 1462-1466

Pitts RJ, Cernac A, Estelle M (1998) Auxin and ethylene promote root hair elongation in Arabidopsis. Plant Journal 16: 553-560

Plant AL, Covey SN, Grierson D (1985) Detection of a subgenomic messenger-RNA for gene-V, the putative reverse-transcriptase gene of Cauliflower mosaic-virus. Nucleic Acids Res 13: 8305-8321

Pooggin M, Hohn T (2003) RNAi targeting of DNA virus in plants. Nature Biotechnology 21: 131-132

Pooggin MM, Hohn T, Futterer J (2000) Role of a short open reading frame in ribosome shunt on the cauliflower mosaic virus RNA leader. Journal of Biological Chemistry 275: 17288-17296

Pooggin MM, Futterer J, Skryabin KG, Hohn T (2001) Ribosome shunt is essential for infectivity of cauliflower mosaic virus. Proc Natl Acad Sci U S A 98: 886-891

Pooma W, Gillette WK, Jeffrey JL, Petty ITD (1996) Host and viral factors determine the dispensability of coat protein for bipartite geminivirus systemic movement. Virology 218: 264-268

Qiu SG, Wintermantel WM, Sha Y, Schoelz JE (1997) Light-dependent systemic infection of solanaceous species by cauliflower mosaic virus can be conditioned by a viral gene encoding an aphid transmission factor. Virology 227: 180-188

Qiu W, Park JW, Scholthof HB (2002) Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. Mol Plant microbe Interactions 15: 269-280

Qu F, Morris TJ (2005) Suppressors of RNA silencing encoded by plant viruses and their role in viral infections. Febs Letters 579: 5958-5964

Rajagopal R (1977) Effect of Tobacco mosaic-virus infection on endogenous levels of indoleacetic, phenylacetic and abscisic acids of tobacco-leaves in various stages of development. Zeitschrift fur Pflanzenphysiologie 83: 403-409

Ramos JA, Zenser N, Leyser O, Callis J (2001) Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. Plant Cell 13: 2349-2360

Ratcliff F, Harrison BD, and Baulcombe DC (1997) A similarity between viral defense and gene silencing in plants. Science 276: 1558-1560

Raven (1975) Transport of indoleacetic acid in plant cells in relation to pH and electrical potential gradients and its significance for polar transport, New Phytologist 74: 163-172

Ravnikar M, Gogala N (1990) Regulation of potato meristem development by jasmonic acid *in vitro*. Journal of Plant Growth Regulation 9: 233-236

Raz V, Ecker JR (1999) Regulation of differential growth in the apical hook of Arabidopsis. Development 126: 3661-3668

Reddy VS, Ali GS, Reddy ANS (2002) Genes Encoding Calmodulin-binding Proteins in the *Arabidopsis* Genome. J Biol Chem 277: 9840-9852

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Current Opinion in Plant Biology 1: 404-411

Roberts AG, Cruz SS, Roberts IM, Prior DAM, Turgeon R, Oparka KJ (1997) Phloem unloading in sink leaves of Nicotiana benthamiana: Comparison of a fluorescent solute with a fluorescent virus. Plant Cell 9: 1381-1396

Roberts AG, Oparka KJ (2003) Plasmodesmata and the control of symplastic transport. Plant Cell and Environment 26: 103-124

Rodriguez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleecker AB (1999) A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science 283: 996-998

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR (1995) Genetic-analysis of ethylene signal-transduction in *Arabidopsis thaliana* - 5 novel mutant loci integrated into a stress-response pathway. Genetics 139: 1393-1409

Ross J, O'Neill D (2001) New interactions between classical plant hormones. Trends in Plant Science 6: 2-4

Ross JJ, O'Neill DP, Smith JJ, Kerckhoffs LHJ, Elliott RC (2000) Evidence that auxin promotes gibberellin A(1) biosynthesis in pea. Plant Journal 21: 547-552

Rothnie HM, Chapdelaine Y, Hohn T (1994) Pararetroviruses and retroviruses - a comparative review of viral structure and gene-expression strategies. Adv Virus Res 44: 1-67

Rouse D, Mackay P, Stirnberg P, Estelle M, Leyser O (1998) Changes in auxin response from mutations in an AUX/IAA gene. Science 279: 1371-1373

Rubery PH (1990) Phytotropins: Receptors and endogenous ligands. Symp. Soc. Exp. Biol. 44: 119–146

Rubery PH, Sheldrake AR (1974) Carrier-mediated auxin transport. Planta 118: 101-121

Ruegger M, Dewey E, Hobbie L, Brown D, Bernasconi P, Turner J, Muday G, Estelle M (1997) Reduced naphthylphthalamic acid binding in the tir3 mutant of Arabidopsis is associated with a reduction in polar auxin transport and diverse morphological defects. Plant Cell 9: 745-757

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. Genes & Development 12: 198-207

Ryabov EV, Roberts IM, Palukaitis P, Taliansky M (1999) Host-specific cell-to-cell and long-distance movements of cucumber mosaic virus are facilitated by the movement protein of groundnut rosette virus. Virology 260: 98-108

Ryabova L, Park HS, Hohn T (2004) Control of translation reinitiation on the cauliflower mosaic virus (CaMV) polycistronic RNA. Biochem Soc Trans 32: 592-596

Ryabova LA, Hohn T (2000) Ribosome shunting in the cauliflower mosaic virus 35S RNA leader is a special case of reinitiation of translation functioning in plant and animal systems. Genes & Development 14: 817-829

Sakai H, Hua J, Chen QHG, Chang CR, Medrano LJ, Bleecker AB, Meyerowitz EM (1998) ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. Proc Natl Acad Sci U S A 95: 5812-5817

Samuel G (1934) The movement of tobacco mosaic virus within the plant. Annals of Applied Biology 21: 90-111

Schaller F, Schaller A, Stintzi A (2004) Biosynthesis and metabolism of jasmonates. Journal of Plant Growth Regulation 23: 179-199

Schoelz J, Shepherd RJ, Daubert S (1986) Region-VI of cauliflower mosaic-virus encodes a host range determinant. Mol Cell Biol 6: 2632-2637

Schoelz JE, Daubert SD, Shepherd RJ (1984) Gene-VI of Cauliflower mosaic-virus (CaMV) controls systemic spread in Solanaceous hosts. Phytopathology 74: 861

Schoelz JE, Shepherd RJ (1988) Host range control of cauliflower mosaic-virus. Virology 162: 30-37

Scholthof HB, Gowda S, Wu FC, Shepherd RJ (1992) The full-length transcript of a caulimovirus is a polycistronic messenger-RNA whose genes are trans activated by the product of gene-VI. J Virol 66: 3131-3139

Scholthof HB, S Scholthof KBG, Kikkert M, Jackson AO (1995) Tomato bushy stunt virus spread is regulated by 2 nested genes that function in cell-to-cell movement and host-dependent systemic invasion. Virology 213: 425-438

Scholthof HB (2005) Plant virus transport: motions of functional equivalence. Trends in Plant Science 10: 376-382

Sequeira L (1973) Hormone metabolism in diseased plants. Annual Review of Plant Physiology and Plant Molecular Biology 24: 353-380

Sheng JS, Lartey R, Ghoshroy S, Citovsky V (1998) An *Arabidopsis* thaliana mutant with virus-inducible phenotype. Virology 249: 119-128

Shirasu K, Schulze-Lefert P (2003) Complex formation, promiscuity and multi-functionality: protein interactions in disease-resistance pathways. Trends in Plant Science 8: 252-258

Silhavy D, Molnár A, Lucioli A, Szittya G, Hornyik C, Tavazza M, Burgyán J (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. The EMBO Journal 21: 3070-3080

Singh DP, Moore CA, Gilliland A, Carr JP (2004) Activation of multiple antiviral defence mechanisms by salicylic acid. Mol Plant Pathol 5: 57-63

Smith SII, McCall SR, Harris JII (1968) Alterations in auxin levels of resistant and susceptible hosts induced by curly top virus. Phytopathology 58: 575-&

Solano R, Ecker JR (1998) Ethylene gas: perception, signaling and response. Current Opinion in Plant Biology 1: 393-398

Staswick PE, Tiryaki I, Rowe ML (2002) Jasmonate response locus JAR1 and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell 14: 1405-1415

Stavolone L, Villani ME, Leclerc D, Hohn T (2005) A coiled-coil interaction mediates cauliflower mosaic virus cell-to-cell movement. Proc Natl Acad Sci U S A 102: 6219-6224

Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CJ, Paris S, Gäleiler L, Palme K, Jurgens G (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science 286: 316-318

Stepanova AN, Ecker JR (2000) Ethylene signaling: from mutants to molecules. Current Opinion in Plant Biology 3: 353-360

Storms MMH, Kormelink R, Peters D, vanLent JWM, Goldbach RW (1995) The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology 214: 485-493

Strange RN, Scott PR (2005) Plant disease: A threat to global food security. Annual Review of Phytopathology 43: 83-116

Stratford R, Covey SN (1989) Segregation of cauliflower mosaic virus symptom genetic determinants. Virology 172: 451-459

Swarup R, Marchant A, Bennett MJ (2000) Auxin transport: providing a sense of direction during plant development. Biochem Soc Trans 28: 481-485

Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the Arabidopsis root apex. Genes & Development 15: 2648-2653

Swarup R, Parry G, Graham N, Allen T, Bennett M (2002) Auxin cross-talk: integration of signalling pathways to control plant development. Plant Mol Biol 49: 411-426

Swarup R, Kargul J, Marchant A, Zadik D, Rahman A, Mills R, Yemm A, May S, Williams L, Millner P, Tsurumi S, Moore I, Napier R, Kerr ID, Bennett MJ (2004) Structure-function analysis of the presumptive Arabidopsis auxin permease AUX1. Plant Cell 16: 3069-3083

Sziraki I, Balazs E, Kiraly Z (1980) Role of different stresses in inducing systemic acquired resistance to TMV and increasing cytokinin levels in tobacco. Physiol. Plant Pathology 16: 277-284

Takahashi H, Kobayashi T, Sato-Nara K, Tomita K, Ezura H (2002) Detection of ethylene receptor protein Cm-ERS1 during fruit development in melon (Cucumis melo L.). J Exp Bot 53: 415-422

Taliansky M, Roberts IM, Kalinina N, Ryabov EV, Raj SK, Robinson DJ, Oparka KJ (2003) An umbraviral protein, involved in long-distance RNA

movement, binds viral RNA and forms unique, protective ribonucleoprotein complexes. J Virol 77: 3031-3040

Tamai A, Meshi T (2001) Cell-to-cell movement of potato virus X: The role of p12 and p8 encoded by the second and third open reading frames of the triple gene block. Mol Plant-Microbe Interact 14: 1158-1167

Thomas CL, Perbal C, Maule AJ (1993) A mutation of Cauliflower mosaic-virus gene-I interferes with virus movement but not virus-replication. Virology 192: 415-421

Thomas CL, Maule AJ (1995) Identification of Structural Domains within the Cauliflower Mosaic Virus Movement Protein by Scanning Deletion Mutagenesis and Epitope Tagging. The Plant Cell 7: 561-572

ThomasCL, Maule AJ (1995) Identification of the Cauliflower mosaic-virus movement protein RNA-binding domain. Virology 206: 1145-1149

Thomas CL, Maule AJ (1999) Identification of inhibitory mutants of Cauliflower mosaic virus movement protein function after expression in insect cells. J Virol 73: 7886-7890

Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate jasmonate-dependent and

salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci U S A 95: 15107-15111

Thomma BPHJ, Eggermont K, Tierens KFMJ, Broekaert WF (1999) Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by Botrytis cinerea. Plant Physiol 121: 1093-1101

Tiryaki I, Staswick PE (2002) An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signalling mutant axr1. Plant Physiology 130: 887-894

Torrance L, Cowan GH, Gillespie T, Ziegler A, Lacomme C (2006) Barley stripe mosaic virus-encoded proteins triple-gene block 2 and gamma b localize to chloroplasts in virus-infected monocot and dicot plants, revealing hitherto-unknown roles in virus replication. J Gen Virol 87: 2403-2411

Torruella M, Gordon K, Hohn T (1989) Cauliflower mosaic-virus produces an aspartic proteinase to cleave its polyproteins. EMBO J 8: 2819-2825

Traynor P, Young BM, Ahlquist P (1991) Deletion analysis of brome mosaic virus 2a protein: effects on RNA replication and systemic spread. J. Virology 65: 2807-2815

Tsuge S, Kobayashi K, Nakayashiki H, Mise K, Furusawa I (1999) Cauliflower mosaic virus ORF III product forms a tetramer in planta: Its implication in viral DNA folding during encapsidation. Microbiol Immunol 43: 773-780

Turner DS, McCallum DG, Covey SN (1996) Roles of the 35S promoter and multiple overlapping domains in the pathogenicity of the pararetrovirus cauliflower mosaic virus. J Virol 70: 5414-5421

Ulmasov T, Murfett J, Hagen G, Guilfoyle T (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9: 1963 1971.

van Loon LC, Geraats BPJ, Linthorst HJM (2006) Ethylene as a modulator of disease resistance in plants. Trends in Plant Science 11: 184-191

Vance V, Vaucheret H (2001) RNA silencing in plants - Defense and counterdefense. Science 292: 2277-2280

Van Loon LC (1979) Effects of auxin on the localization of Tobacco mosaic-virus in hypersensitively reacting tobacco. Physiol. Plant Pathology 14: 213-226

Voinnet O, Pinto YM, Baulcombe DC (1999) Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. Proc Natl Acad Sci U S A 96: 14147-14152

Voinnet O (2001) RNA silencing as a plant immune system against viruses. Trends in Genetics 17: 449-459

Waigmann E, Ueki S, Trutnyeva K, Citovsky V (2004) The ins and outs of nondestructive cell-to-cell and systemic movement of plant viruses. Crit Rev Plant Sci 23: 195-250

Wang HL, Wang Y, Giesman-Cookmeyer D, Lommel SA, Lucas WJ (1998) Mutations in viral movement protein alter systemic infection and identify an intercellular barrier to entry into the phloem long-distance transport system. Virology 245: 75-89

Ward BM, Medville R, Lazarowitz SG, Turgeon R (1997) The geminivirus BL1 movement protein is associated with endoplasmic reticulum-derived tubules in developing phloem cells. J Virol 71: 3726-3733

Weaver LM, Gan S, Quirino B, Amasino RM (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol Biol 37: 455-469

Weiland JJ, Edwards MC (1996) A single nucleotide substitution in the alpha a gene confers oat pathogenicity to barley stripe mosaic virus strain ND18. Mol Plant-Microbe Interact 9: 62-67

Went FW (1926) Concerning the difference in sensibility of the tip and base of Avena to light. Proceedings of the Koninklijke Akademie Van Wetenschappen Te Amsterdam 29: 185-191

Whitham SA, Quan S, Chang HS, Cooper B, Estes B, Zhu T, Wang X, Hou Y M (2003) Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. Plant J. 33:271-283.

Wintermantel WM, Anderson EJ, Schoelz JE (1993) Identification of domains within gene VI of Cauliflower mosaic virus that influence systemic infection of *Nicotiana bigelovii* in a light-dependent manner. Virology 196: 789-798

Wojtaszek P (1997) Oxidative burst: An early plant response to pathogen infection. Biochemical Journal 322: 681-692

Wolf S, Deom CM, Beachy RN, Lucas WJ (1989) MOVEMENT PROTEIN OF TOBACCO MOSAIC-VIRUS MODIFIES PLASMODESMATAL SIZE EXCLUSION LIMIT. Science 246: 377-379

Worley CK, Zenser N, Ramos J, Rouse D, Leyser O, Theologis A, Callis J (2000) Degradation of Aux/IAA proteins is essential for normal auxin signalling. Plant Journal 21: 553-562

Xie Q, Guo HS (2006) Systemic antiviral silencing in plants. Virus Research 118: 1-6

Xie Z, Fan B, Chen C, Chen Z (2001) An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defence. Pro Natl Acad Sci USA 98: 6516-6521

Xin HW, Ding SW (2003) Identification and molecular characterization of a naturally occurring RNA virus mutant defective in the initiation of host recovery. Virology 20: 253-262

Xu XZ, Wes PD, Chen H, Li HS, Yu M, Morgan S, Liu Y, Montell C (1998) Retinal targets for calmodulin include proteins implicated in synaptic transmission. J Biol Chem 273: 31297-31307

Yu WC, Murfett J, Schoelz JE (2003) Differential induction of symptoms in Arabidopsis by P6 of Cauliflower mosaic virus. Mol Plant-Microbe Interact 16: 35-42

Zhang H, Zhu X, Liu H (1997) Effect of banana bunchy top virus (BBTV) on endogenous hormone of banana plant. Acta Phytopathologica Sinica 27: 79-83

Zhao MM, An DR, Huang GH, He ZH, Chen JY (2005) A viral protein suppresses siRNA-directed interference in tobacco mosaic virus infection. Acta Biochim Biophys Sin (Shanghai) 37: 248-53

Zhou XP, Liu YL, Calvert L, Munoz C, OtimNape GW, Robinson DJ, Harrison BD (1997) Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J Gen Virol 78: 2101-2111

Zijlstra C, Hohn T (1992) Cauliflower mosaic virus gene VI controls translation from dicistronic expression units in transgenic Arabidopsis plants. Plant Cell 4: 1471-1484

Zijlstra C, ScharerHernandez N, Gal S, Hohn T (1996) *Arabidopsis thaliana* expressing the cauliflower mosaic virus ORF VI transgene has a late flowering phenotype. Virus Genes 13: 5-17

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis Microarray Database and Analysis Toolbox. Plant Physiology 136: 2621-2632.

Appendices

Apendix 7.0

Disc 7.0 Statistics

Virus Accumulation (7.1)

7.1.1 Col-0, *aux1-7* and *aux1-22*

7.1.2 Ws and *aux1-100*

7.1.3 *tir* mutants and Col-0

Auxin Responsive Gene Expression (7.2)

7.2.1 Col-0, *aux1-7* and *aux1-22*

7.2.2 Ws and *aux1-100*

7.2.3 *tir* mutants and Col-0

Genetic Analysis (7.3)

7.3.1 *tir3-1* and Col-0 x A7